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Microbial ecology of Intermittently Aerated Sequencing Batch Reactors (IASBRs) for the treatment of dairy processing wastewaters

Thesis presented by

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Declaration of independence

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of Univeristy College Cork concerning plagiarism.

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Beatriz Gil Pulido

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“If you want to go fast, go alone. If you want to go far, go together”

(African Proverb)

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To my grandfather Pepe and my friend Gloria.

Abstract

This thesis was conducted as part of the Dairy Water Project, funded by the Department of Agriculture, Food and the Marine, Ireland (Ref.: 13-F-507). The Dairy Water project was a multi-stakeholder research collaboration focussed on evaluating opportunities to increase environmental sustainability within the Irish Dairy processing sector. The research scope of the Dairy Water project included: water consumption in the Irish dairy industry and potential water re-use and/or rainwater harvesting; nanomaterial based disinfection technologies and dairy wastewater characterisation; secondary treatment via Intermittently Aerated Sequencing Batch Reactor (IASBR) technology and microbial ecological profiling of systems from laboratory to pilot-scale. In this context, the work presented in this thesis focuses on the microbial characterization of the IASBR technology applied to dairy processing wastewaters bioremediation, which was operated by engineering collaborators in the National University of Ireland, Galway.

Ireland is one of Europe's largest producers and exporter of milk and the dairy processing industry is a key component of the national economy. Following abolition of the European milk quotas in 2015, the dairy industry in Ireland significantly increased its milk production and exports annually. However, processing of milk into secondary products, (e.g. ingredients, cheeses, milk powders, etc.), generates significant volumes of high nutrient-load wastewaters. These require extensive remediation prior to release into natural environments to meet permitted discharge license limits regulated by the Environmental Protection Agency. Traditional biological treatments are often applied to dairy processing wastewater remediation, but typically require chemical precipitant use, high energy inputs and separate bioreactor units with significant infrastructural/capital demand. As a result, there is a continual drive within the wastewater treatment sector to develop cost-effective, high performance, low footprint technologies. IASBR systems are an emerging technology offering an economical and sustainable solution for co-nutrient removal in a single reactor. IASBRs have been successfully applied at laboratory scale to the treatment of domestic and slaughterhouse wastewaters, however the nature of the microbial ecology of these systems is

poorly understood. In biological wastewater treatments, microorganisms are the key players and the success in removing pollutants from wastewaters is dependent on their capabilities to remediate such substances. Hence, knowledge of the bacterial community (e.g. overall diversity, stability, dominant relative abundances etc.) in the different remediation processes may contribute to optimisation of bioreactor performance.

The study presented here seeks to address the existing knowledge gap in relation to the microbial community structure of IASBRs via next generation sequencing approaches (454 pyrosequencing, MiSeq). Furthermore, high throughput sequencing techniques were combined with comprehensive *in silico* and statistical analyses to assess the functional gene diversity and dynamics of bacterial populations within representative biomass samples in laboratory and pilot-scale settings. Such knowledge is critical to the understanding and optimisation of IASBR technologies for application to the dairy processing sector and potentially in the treatment of other industrial wastewaters. To position this work within the broader context of wastewater biological treatment technologies, **Chapter 1** presents an overview of the scope and scale of dairy industry and wastewater generation, traditional biological treatment design and operation, emerging technologies, key microbial phyla and the biochemical capacities being exploited and modern molecular approaches to profiling same within mixed culture reactor systems.

In **Chapter 2,** the microbial community structure of a laboratory-scale IASBR system operating at 11˚C was analysed using 454-pyrosequencing technique and *in silico* analyses. The microbial ecology of the IASBR receiving synthetic wastewater was linked with nutrient removal performance within the bioreactor under three different aeration rates, 0.4, 0.6 and 0.8 Litres of oxygen per minute (LPM). In addition, metabolic profiles of the bacterial community were analysed to predict the contribution to nitrogen and phosphorus genes and to identify potential, key contributors to nutrient bioremediation. A key finding was the strong dominance of the family *Comamonadaceae* (>80% relative abundance) in parallel with optimal nitrogen and phosphorus removal efficiencies over 90% during the aeration of 0.6 LPM, which was not maintained at 0.4 or 0.8 LPM aeration rates. **Chapter 3** compared the microbial ecology of two laboratory-scale IASBRs

when they were fed with synthetic and industrial wastewaters, respectively. The bioreactors were initially fed with biomass from an industrial plant and were operated under 0.6 LPM and 11˚C. Metagenomic studies were also combined with comprehensive *in silico* analyses to assess the functional gene diversity within respective biomass samples. In order to gain a greater understanding of population dynamics, statistical analyses were applied to evaluate the impact of wastewater type (synthetic Vs. industrial) on observed communities by means of multivariate redundancy analyses (RDA). Taxonomical analyses revealed the dominance of the *Comamonadaceae* family and members under controlled conditions (synthetic). However, under industrial wastewater influent feeding, bacterial diversity was observed to be more distributed among *Comamonadaceae* and other different families. Functional gene prediction analyses carried out in Chapter 2 and Chapter 3, revealed *Comamonadaceae* family and members as key contributors of nitrogen and phosphorus metabolism genes (*nirK, nosZ, norB, ppK, ppX and phaC*) during laboratory-scale trials. The investigations carried in Chapter 2 and Chapter 3 of the current thesis provide theoretical support for the currently emerging profile in the literature of *Comamonadaceae* members as potentially significant contributors to nitrogen and phosphorus remediation processes in the wastewater sphere, under controlled conditions.

In **Chapter 4**, the gained knowledge on microbial communities of laboratory-scale IASBR system was expanded with the investigation of the bacterial ecology structure and dynamics in a pilot-scale IASBR located at an Irish dairy processing plant operated over a five-month period. Metagenomic 16S rRNA gene analyses of pilot scale IASBR reactor samples via Illumina Miseq sequencing revealed a more complex and diverse bacterial community profile in the pilot-scale IASBR than those observed previously in laboratory-scale settings. Although some of the predominant phyla and orders identified were shared with the systems reported in Chapter 2 and Chapter 3, such as *Bacteroidetes* and *Proteobacteria* phyla, a number of distinct bacterial groups were observed in the pilot-scale system. Interestingly, the main difference observed at pilot-scale was the absence of *Comamonadaceae* family and members. Overall, the composition of the bacterial community in the pilot-scale system operated over an extended period was stable in parallel with

high nutrient removal performance within the bioreactor (>95% for both nitrogen and phosphorus).

The three different trial systems investigated in the current thesis displayed differing bacterial community profiles despite consistent optimal nutrient performance. Thus, IASBR reactors, as operated in the current study, do not appear to be strictly dependent on the dominance of any particular genus for high performance. This demonstrates the potential versatility in diverse wastewater remediation applications for this biological treatment technology.

Chapter 1

Dairy industry processing wastewaters: Biological treatments for nitrogen and phosphorus removal.

Literature Review

1.1 Overview of the global dairy industry

Milk production and processing into a large variety of fresh and manufactured products play a major part in the agri-food sector across the globe. The dairy industry is currently undergoing significant, global expansion driven by population growth and increased demand for dairy products such as fresh milk, skimmed milk powder and cheese. According to the latest figures published by the Organisation for Economic Co-operation and Development (OECD) world milk production is projected to increase by 22% in 2027 compared to the 2015-17 base period (OECD, 2018). In 2030, world milk production is expected to reach more than 1 billion litres (EC, 2017a). Much of the predicted increase is targeted at fresh dairy products, with the majority of the demand coming from developing countries (Figure 1). It is anticipated that India will account for the greatest increase in milk production during the period 2018-2027 positioning the country as the major milk producer surpassing the European Union (EU) with a global share of 25% compared to the 18% expected for the EU in 2027 (OECD, 2018).

Following the abolition of the milk quotas in the EU at the end of March 2015, total milk production increased in several member states including Ireland (18.5%), the Netherlands (11.9%) and Germany (2.9%) for the period 2015-2016 (OECD, 2016). Milk production in farms in the EU in 2016 was approximately 168 million tons (Eurostat, 2017). Predictions for milk production in the EU during the period 2018-2027 estimate <1% growth per year (OECD, 2018). Weather and market conditions could explain these conservative EU forecasts, which strongly influence milk yield (EC, 2017a).

International exportations of butter, cheese, whole milk powder and skim milk powder are expected to grow by 1 million tonnes of milk equivalent per year on average (EC, 2017a) with developed countries accounting for 82 % of the world exports for dairy products by 2027 (OECD, 2018). The European Union is one of the major exporters together with New Zealand and United States (Figure 2) mainly of cheese and skim milk powder. The dairy processing industry in EU brings more than €10 billion to the overall EU trade balance mainly due to a stable and high quality milk supply (EDA, 2017). An increase by more than 400,000 tonnes of milk equivalent per year on average is

predicted according to the latest figures of the European Commission (EC, 2017a). In parallel to the EU exports, the European Commission also outlines that around 900,000 tons of additional milk per year would be needed to satisfy the growth in EU domestic use for dairy products as for example cheese (EC, 2017a).

The dairy sector contributes significantly to regional and national economies and there is an expected expansion of the dairy industry worldwide. However, the quantity of milk produced over time depends on several factors that will affect the future expansion of the dairy industry. For example, changes in domestic policies and in trade agreements could have major impacts in the dairy market. However, environmental legislation and sustainability goals will also likely play a major role in shaping milk production development and the dairy market (EC, 2017a; OECD, 2018).

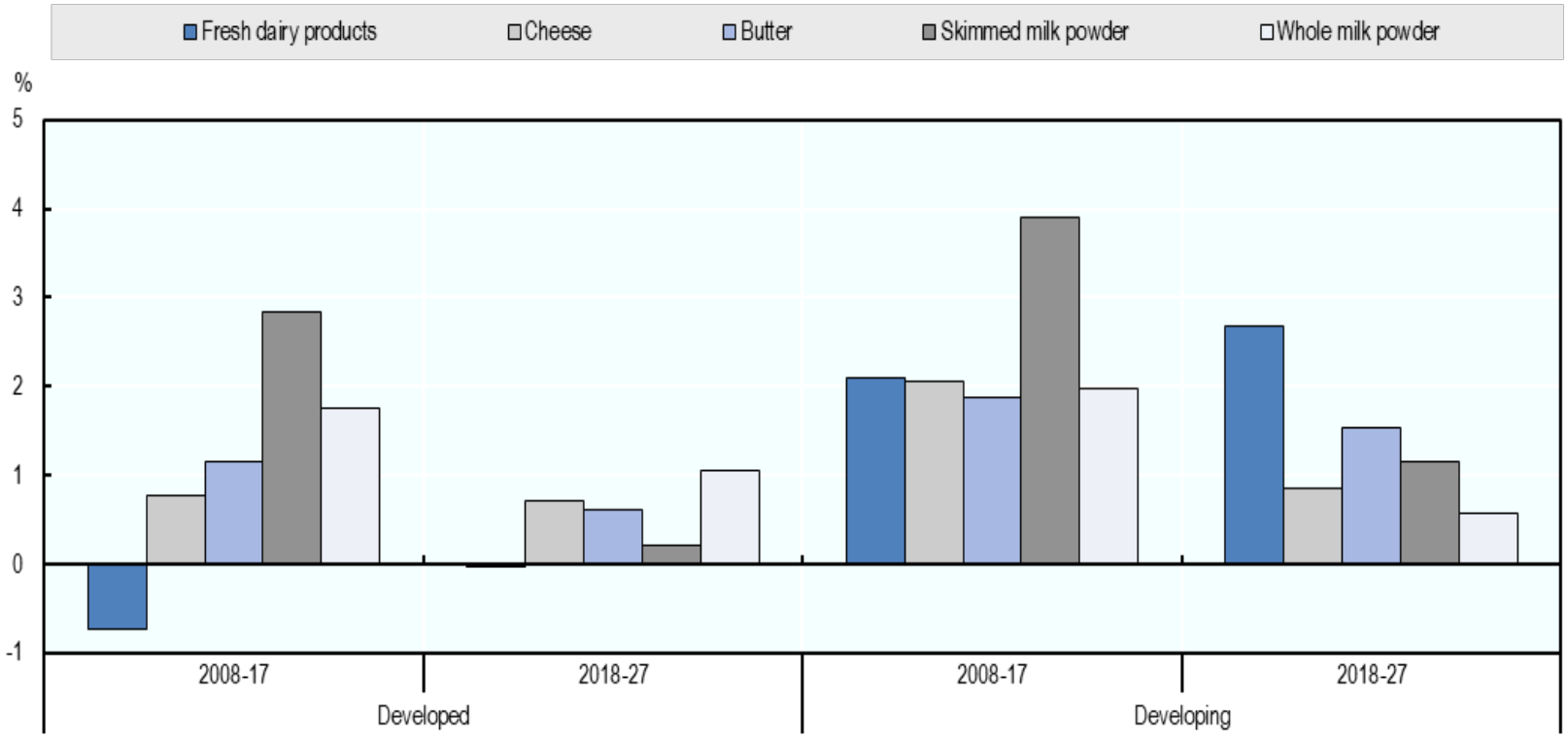

Figure 1. Annual growth rates per capita consumption of dairy products (OECD, 2018).

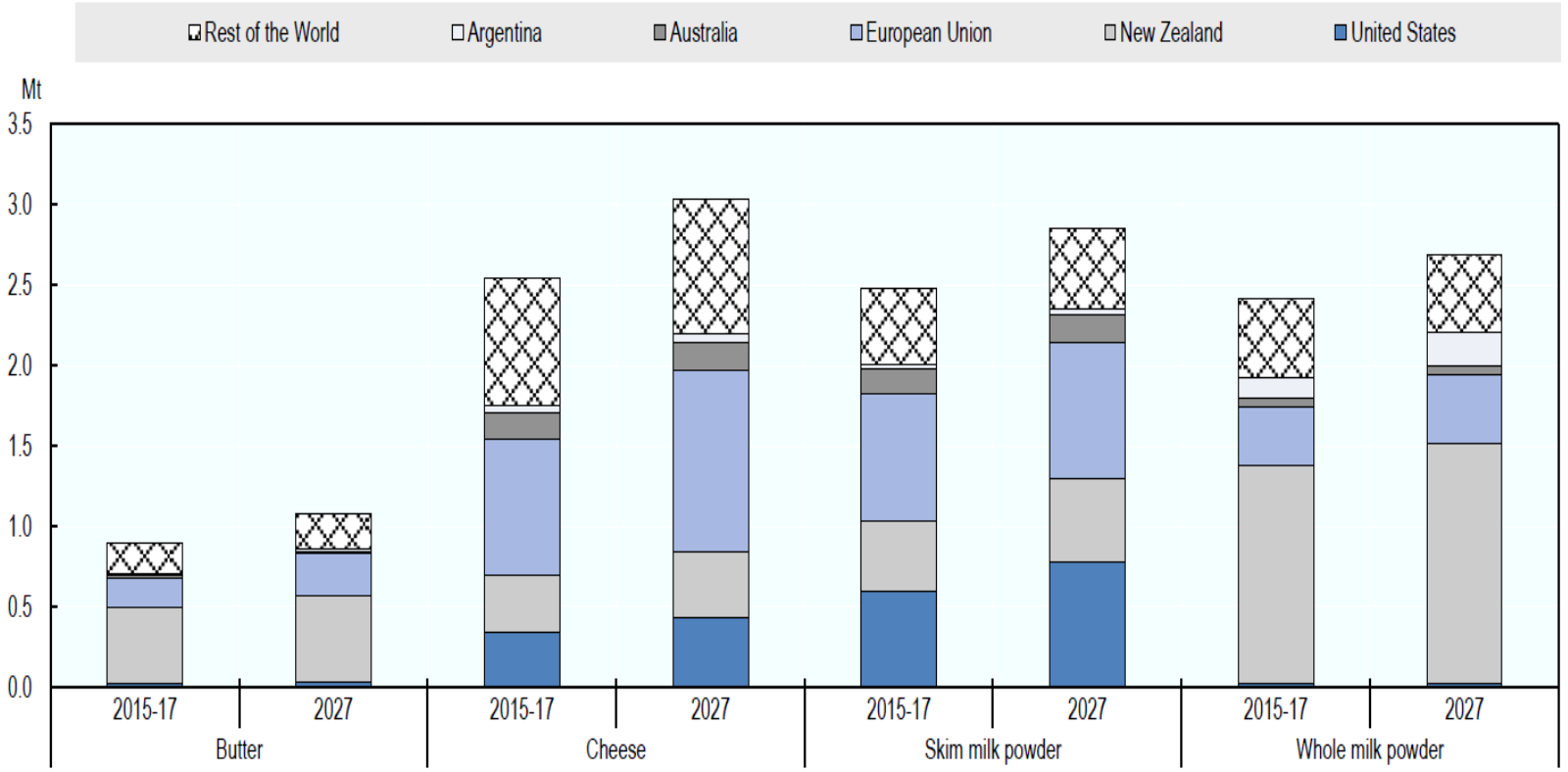

Figure 2. Exports of dairy products by region (OECD, 2018).

1.2 The Dairy processing sector in Ireland

Ireland is one of the major producers and exporters of milk in the EU and the dairy industry is a key component of the national economy (DAFM, 2015; Bord Bia, 2017; Teagasc, 2018). As a result of the European milk quota system abolition in 2015, which had placed limits on milk production since the mid 1980's, milk production in Ireland experienced a strong period of growth (Figure 3). In 2018, there was an increase of 4.3% in Irish milk production (CSO, 2018). Competitive milk prices, increases in dairy herd numbers and good grass conditions during 2017 also contributed to this expansion (Teagasc, 2018). Growth has continued into 2019 with an 8.6% increase in the first quarter when compared with 2018 figures (CSO, 2019).

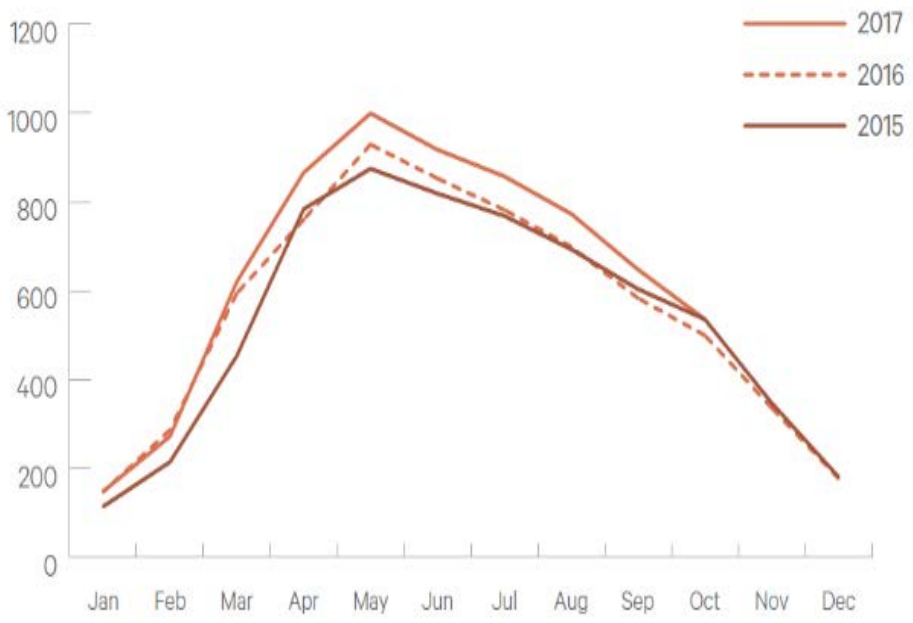

Figure 3. Irish Milk Production for the period 2015-2017 (million litres) (Bord Bia, 2017).

Dairy processing plants perform a range of milk handling operations for the manufacture of a large variety of products. Approximately 93% of the milk produced in Ireland is processed into a number of different dairy products, including cheese, milk powder and butter (NMA, 2017). To date, dairy products and ingredients consist of 30% of the Irish food and drinks export

market (Finnegan et al., 2018a). The value of Irish dairy exports increased by 19% to €4 billion in 2017 with cheese as the largest dairy export followed by butter (Bord Bia, 2017). High standards of quality in Irish dairy ingredients and products along with grass production systems makes the Irish dairy sector a competitive market in the international sphere. Effective and competitive production in the Irish dairy sector while ensuring the protection of the natural environment has become a major feature within the industry in recent years. The increase in the volume of milk being processed along with stringent measures on emissions standards is challenging the Irish dairy industry to seek innovate technological solutions to make the dairy processing sector more efficient and sustainable (Bord Bia, 2013; Finnegan et al., 2018a).

1.3 Environmental impact of the dairy processing industry. Water consumption

The European BREF document for Food, Drink and Milk Industries reported 12,000 dairy processing plants in the EU (EC, 2017b). The production, processing and distribution of milk and secondary dairy products can impact the environment at various levels and produce waste streams of differing quantity and composition, depending on the nature of the products. As a result, the environmental impact associated with the expanding dairy sector is of particular concern. Several life cycle assessment (LCA) studies performed to date have examined the environmental impacts associated with dairy processing industries and identified energy demand, water consumption and wastewater generation as key environmental issues (EC, 2006; EPA, 2008; Pagan et al., 2010; Geraghty, 2011; Fantin et al., 2012; Finnegan et al., 2015; Finnegan et al., 2018b).

Within the agri-food sector, the dairy industry is considered one of the major consumers of water resources (Pagan et al., 2010; Geraghty, 2011; Rad and Lewis, 2014). Water is used for different operational processes during the manufacture of dairy products and also in cooling, heating and cleaning operations (Figure 4). The largest on-site water demands in a dairy

processing plant typically relate to cleaning-in place (CIP) systems (EPA, 2016; Finnegan et al., 2018a), which are mainly sourced from surface water courses (Finnegan et al., 2015). CIP systems are commonly used in food and drink industries that require high levels of hygiene and consist of passaging rinsing water and a cleaning agent through the pipes, tanks and process lines (Britz et al., 2006; Thomas and Sathian, 2014). CIP systems may account for up to 75% of the plant's total water consumption and can vary depending on the product processed and/or procedural variations at plant level (Finnegan et al., 2018a). For example, CIP systems used during the production of cream can consume 1−7% of plant's total water needs while milk powder consumes 56% of the overall water consumed in the plant (Finnegan et al., 2018a). Additionally, the manufacture of milk powder in two different dairies have reported 33% and 75% water consumption rates, respectively (Finnegan et al., 2018a).

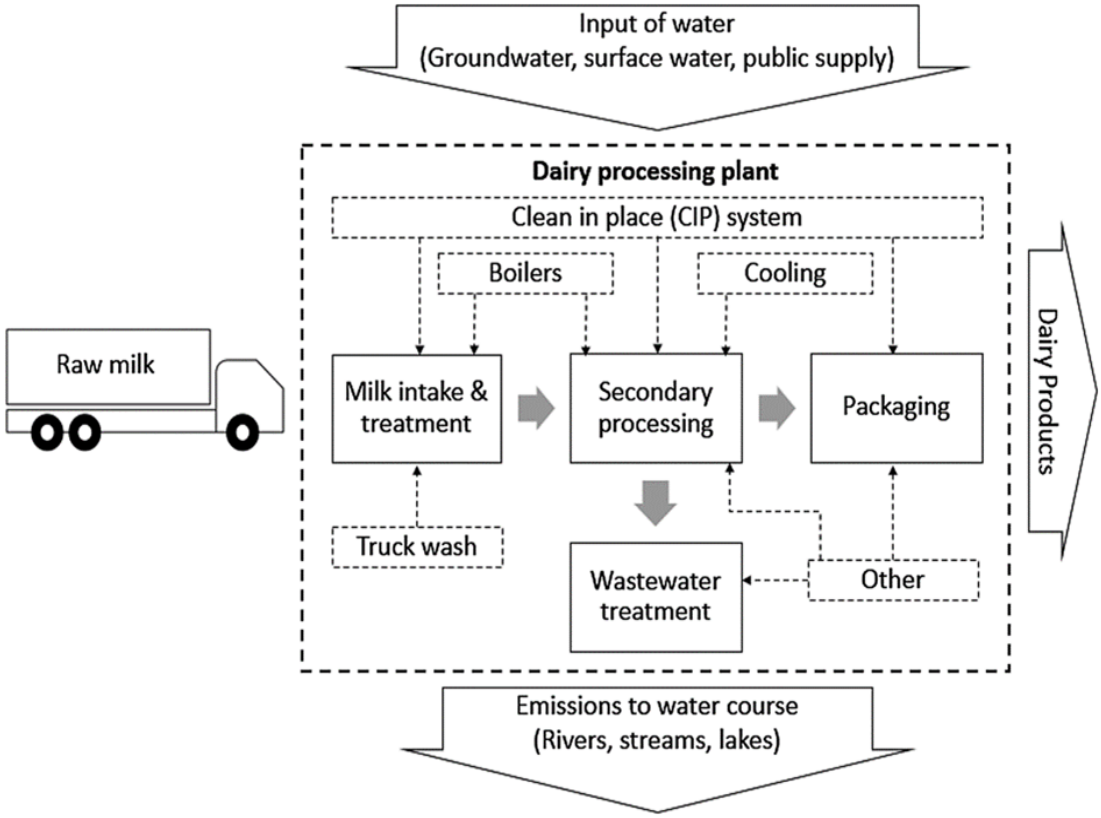

Figure 4. Diagram of the main water users with the manufacture of dairy products. (Finnegan et al., 2018a).

The volume of water consumed in dairies varies considerably. The overall water consumed by 18 Irish dairy plants was reported at 13 billion litres in 2013 (Finnegan et al., 2015). A sample of total water consumption volumes of 4 of the biggest Irish dairy processing plants based on license reports issued by the Environmental Protection Agency (EPA) for the period 2016-2017 are presented in Table 1.

Table 1. Water usage and supply sources of 4 Irish dairy processing factories for the period 2016-2017. Public supply (PS), Groundwater abstraction (GWA), Surface Water Abstraction (SWA)

	Water supply source	**Volume (m^3/yr)**
Site 1	PS + GWA	404,148
Site 2	PS + Other	1,036,455
Site 3	PGW	171,501
Site 4	GWA + SWA +PS + Other	1,576,279

These results show the large variation ranges of water usage data in different dairies driven by different factors associated with operating practices, plant complexity, nature of the water sources and the cost of water (EC, 2006; Rad and Lewis, 2014). Such variation is mirrored in the European dairy industry where water consumption can range between 0.25−8.12 m^3 per tonne of processed raw material according to the figures presented by the European Commission (EC, 2017b). The average estimated water consumption for the period 2003-2009 in the Irish dairy processing sector was 2.5 litres of water per litre of milk processed (Geraghty, 2011). In 2013, there was a slight reduction of 9% compared to the figures in 2009 and dairy processors consumed approximately 2.3 litres of water per litre of milk processed (Finnegan et al. 2015). In a study carried by Eide on the environmental

impact of three Norwegian dairies concluded that the water consumption varied between 1.05 to 1.3 litres of water per litre of drinking milk depending on plant size (Eide, 2002). In addition, the Environmental Protection Agency (EPA) in Ireland, reported that the Aurivo Co-Operative Society Ltd. used 0.75 litres of water per litre of milk processed (EPA, 2016). These examples suggest that greater water consumption efficiency in the dairy industry is possible.

The different phases involved in the production and processing of milk require large inputs of resources such as for example energy and water as outlined in this section. The minimisation of water usage in the dairy processing sector is driven by environmental and economic factors and it is an issue that has been highlighted in the literature (Forfas, 2004; Rad and Lewis, 2014; DAFM, 2015 EPA, 2016). Water consumption minimisation can be achieved by equipment improvement or by technological means, which can lead to significant cost savings to the industry (Prasad et al., 2005; Rad and Lewis, 2014, EPA, 2016). For example, National Foods Ltd (Sydney, Australia) reduced water use at its plant by 22% (110,000 litres/day) by moving to a more efficient pasteuriser and bottle washing system, among other potential improvements made, and saving AUS $104,000 per year (Prasad et al., 2014). In Ireland, the EPA (2016) reported that Aurivo Dairy achieved a 34% reduction in water consumption using improved wash cycle control among other improvements on site, while milk processing on site increased by 47% in the same period, (Daly, 2013; EPA, 2016). Water consumption minimisation is directly related to the improvement of the environmental impact of the dairy processing industry and it represents and increasing challenge for dairy processors

1.4 Dairy processing wastewater

The combination of high levels of water consumption in dairy processing plants together with the inherent production of liquid by-product from milk processing, results in the dairy industry generating significant volumes of wastewater per unit of product processed (Brião and Tavares, 2007; Shete and Shinkar., 2013a; EPA 2016). These effluents are generated from

different sources during milk processing as shown in Figure 5 (Kushwaha et al., 2011) and can be divided into three different categories: process water, cleaning water and sanitary water (Britz et al., 2006; Kolev Slavov, 2017). The largest amount of wastewater is generated from cleaning operations particularly when different products are produced in a single production unit (Shete and Shinkar, 2013b; Rad and Lewis 2014). On-site cleaning operations are undertaken at the end of each production process with intermittent washing activities ongoing during the manufacturing process.

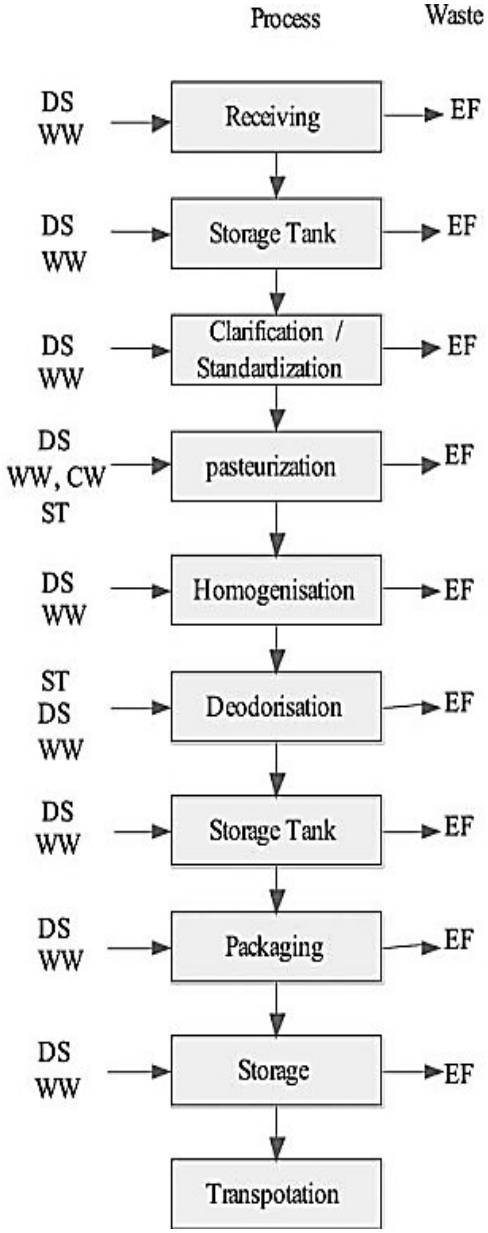

Figure 5. Diagram of different effluent sources during milk processing. Effluent (EF); Detergents and sanitizers (DS); Wash water (WW); Steam (ST); Cooling water (CW). (Kushwaha et al., 2011).

The volume of processing wastewater generated in the dairy industry is highly variable and depends on several factors such as the volume of milk processed, product manufacture and plant size and management strategies. These effluents are intermittently generated and show seasonal flow variations. A typical European dairy processing plant is estimated to produce around 500 m3 of wastewater per day but the volume is highly variable and relates to the volume of product processed (Demirel et al., 2005). Wastewater volumes produced in plants processing several dairy products can range from 0.2 to 10 litres per litre or kilogram of milk processed (Veysseyre, 1988; Braile and Cavalcanti, 1993; UNEP 2004; EC, 2006; Vourch et al., 2008; EC, 2017a,2017b.) (Table 2)

Table 2. Dairy wastewater generation for the processing of different dairy products.

Product	***Volume***	***Reference***
Several products	1.1 −6.8 L/L milk	Braile and Cavalcanti (1993)
Several products	7−10 L/L milk	Veysseyre (1988)
Several products	0.2−10 L/L milk	Vourch et al. (2008)
Milk and yogurt	0.9−25 L/kg product	EC (2006)
Cheese	0.78−6.20 L/kg product	EC (2017b)
Powder (different)	1.21−2.95 L/kg raw material	EC (2017b)
Cheese	15.3 L/kg product	EC (2017b)
Milk powder	0.66−2.47 L/kg product	UNEP 2004

Cheese production in European dairies can generate 0.78−6.20 litres of wastewater per kilogram of processed raw material while the production of powder products average from 1.21−2.95 litres of wastewater per kilogram (EC, 2017b). Values can be significantly higher, as per one example of an Irish cheese manufacture plant which has been reported to generate 15.3 litres of wastewater per kilogram of cheese produced (EC, 2017b). Several authors recommend a benchmarking value of 1 litre of wastewater per litre processed milk for an effective wastewater management in dairies (Carawan and Stengel, 1996; UNEP, 2004; EC, 2006; EA,2009; Nadais et al., 2010). The dairy sector must therefore aim for improved processing methods that minimize the generation of wastewaters if they are to achieve a balance between increased processing capacities and sustainable production.

1.4.1 Composition of dairy effluents

Dairy processing wastewaters are typically high nutrient load effluents although specific compositions vary with each process. In general, dairy waste streams are more concentrated than domestic effluents and are characterized by high loadings of organic matter, nitrogen and phosphorus chemical species. Such effluents would pose a major environmental risk to water bodies if discharged without being properly treated (Britz et al., 2006; Kushwaha et al., 2011; EPA, 2016). Total concentrations of biological oxygen demand (BOD5) up to 4,790 mg L^{-1} and chemical oxygen demand (COD) concentrations of 500−4,500 mg L^{-1} have been reported in untreated dairy effluents (EA, 2009; EAEW, 2000). Nitrogen and phosphorus in dairy wastewaters are significantly higher than in municipal effluents and are mainly sourced from detergents, disinfectants, milk proteins and milk itself (Deremiel et al., 2005; Britz et al., 2006; Broughton et al., 2008). Nitrogen (N) is present in various forms such as ammonia nitrogen (NH_4-N), nitrite (NO_2^-) and nitrate (NO_3^-) (Demirel et al., 2005). Phosphorus is found mainly in inorganic forms such as orthophosphate (PO_4^{3-}), and polyphosphate ($P_2O_4^{-7}$) as well as in organic forms (Srikar Sai, 2000). Mean reported values of total nitrogen (TN) and total phosphorus (TP) loads in dairy processing

wastewaters are around 153 mg L^{-1} and 663 mg L^{-1}, respectively (Cristian, 2010). Total Kjeldahl nitrogen (TKN) concentrations, defined as the total concentration of organic nitrogen and ammonia, of 13-1462 mg L^{-1} and NH_4-N concentrations up to 64.3 mg L^{-1} have been reported in dairy processing wastewaters (Malaspina et al., 1996; Britz et al., 2006). Rule (1997) reported that phosphorus (P) is mainly found as orthophosphate (PO_4^{3-}) accounting for 90% on average of the total P in dairy water streams (Rule, 1997; Guillen-Jimenez et al., 2000). Several authors have reported phosphorus contents up to 125 mg P L^{-1} in dairy wastewaters but wider ranges of 0.2−327 mg P L^{-1} have also been posited (Bickers et al., 2003; Britz et al., 2006). Tables 3.1 and 3.2 summarize the large variation of dairy processing effluent compositions reported in the literature, which are largely dependent on the type of product being processed. Overall, the production of whey and cheese whey produce water streams with the highest concentrations of BOD_5, COD, TKN and TP (Tables 3.1 and 3.2). High BOD_5 values in cheese whey are caused high organic compound loadings, dominated by lactose. Large variations in pH values of dairy processing wastewaters are also possible and relate to cleaning operations in the plant (Kolev Slavov, 2017). Those plants processing whey and/or cheese typically show effluent pH values below 6 (Table 3.1), while pH increases may be due to the discharge of alkaline cleaning solutions (Kolev Slavov, 2017). Typical concentrations of BOD_5, COD, TSS and key nutrients (NH_4-N and PO_4-P) reported for Irish diary processing plants are summarized in Table 4. Finnegan et al., (2018a) surveyed 6 different plants in Ireland and reported significant variation in wastewater composition from site-to site as well as daily variation at a single site (Finnegan et al., 2018a). The authors reported NH_4-N and PO_4^{3-} concentration ranges between 0.9−184.2 mg L^{-1} and 5.0−102 mg L^{-1}, respectively. High variations in COD and BOD_5 concentrations were also observed with maximum values of 9,770 and 4,900 mg L^{-1}, respectively.

Table 3.1. Chemical characteristics of different dairy water streams (adapted from Shete et al., 2013a and Britz et al., 2006).

Product	BOD_5 (mg L^{-1})	COD (mg L^{-1})	pH	TSS (mg L^{-1})	Reference
Cheese & whey	377−2,214	189−6,219	5.2	−	Andreottola et al., 2002
Cheese processing	−	63,300	3.4	12,500	Hwang & Hansen, 1998
Milk & yogurt	−	4,656	6.9	−	Strydom et al., 1997
Milk & Dairy Products	10,251	4,840	8.3	5,802	Cristian, 2010
Whey	35,000	−	4.6	−	Donkin, 1997
Cheese Whey pressed	80,000 − 90,000	120,000 − 135,000	6	8,000 − 11,000	Baroudi et al., 2012

(BOD_5 = Biological Oxygen Demand; COD = Chemical Oxygen Demand; TSS = Total Suspended Solids)

Table 3.2. Nutrient composition of different dairy processing wastewaters (adapted from Britz et al., 2006).

Product	TKN (mg L^{-1})	NH_4-N (mg L^{-1})	Total P (mg L^{-1})	PO_4-P (mg L^{-1})	Reference
Cheese & whey	13−172	0.7-28.5	0.2−48	0.2−7.9	Andreottola et al., 2002
Cheese & casein	140	−	85	−	Sparling et al., 2001
Cheese	102	−	45	−	Sparling et al., 2001
Butter & milk powder	70	−	35	−	Donkin, 1997
Whey	1,400	−	640	−	Donkin, 1997
Raw cheese whey	1,462	64.3	379	327	Malaspina et al., 1996

(TKN = Total Kjeldahl Nitrogen; NH_4-N = ammonia nitrogen; P = Phosphorus; PO_4-P= orthophosphate).

Table 4. Dairy processing wastewater characteristics of 6 Irish dairy processing plants (adapted from Finnegan et al., 2018a).

Site No.	BOD_5 (mg L^{-1})	COD (mg L^{-1})	TSS (mg L^{-1})	NH_4-N (mg L^{-1})	PO_4-P (mg L^{-1})
1	4,900	9,770	2,370	7.8	5.3
2	–	1,430	750	0.9	5.0
5	3,300	3,310	915	184.2	8.1
7	1,900	3,270	490	20.1	31.7
8	1,200	1,210	311	6.1	6.6
9	375	1,171	205	1	102

(BOD_5 = Biological Oxygen Demand; COD = Chemical Oxygen Demand; TSS = Total Suspended Solids; NH_4-N = nitrogen ammonia; PO_4-P = orthophosphate).

1.4.2 Effluent emissions and discharge limits

As outlined in previous sections, the ongoing expansion of milk production and dairy processing will lead to an increase in dairy effluent emissions that will need to be managed in line with Industrial Emission Licences (IELs) prior to discharge (OECD, 2018; Teagasc, 2018). Dairy effluents are discharged from on-site wastewater treatment facilities to surface waters (generally rivers) and must not impair the quality of receiving water bodies. The final effluent volume and permissible concentrations that can be released per day are determined by IELs and Integrated Pollution Control (IPC) licenses and those emission limits will depend on the sensitivity of the receiving water bodies (EPA, 2008). Each processing plant must apply for and comply with a wastewater discharge licence to ensure that discharges do not have any significant impact on the receiving water bodies. Discharge regulations differ significantly from plant to plant depending on discharge practices but the

process effluent emission must be in compliance with the existing licence limits.

Following Directive 2010/75/EU of Industrial Emissions (IED, 2010) the emission limit values for dairy processing effluents are based on the Best Available Techniques (BAT) guidelines. BAT Reference Documents (BREFs) constitute a series of guidance documents for different industry sectors within the EU in order to meet requirements with IED and Integrated Pollution Control (IPC) Licences. (EC, 2017b; Stanley et al., 2017). According to the BAT Guidance Note for the dairy processing sector, parameters that must be considered as an indicator of polluting potential to receiving water bodies are BOD_5, COD, total suspended solids (TSS), pH, temperature, phosphorus, nitrogen and chloride (EPA, 2008). Associated emission levels for discharges to surface water are shown in Table 5. The BAT Guidance Note recommends that final emissions should achieve $\geq$ 80% removal of nutrient load and $\geq$90% BOD_5 reduction in relation to influent loads (EPA, 2008). The range of emission limits for BOD_5, total nitrogen (TN) and total phosphorus (TP) in the final effluent are 20-70 mg $^{L-}$1, 10 mg L^{-1} , 5-25 mg L^{-1} and 2-5 mg L^{-1}, respectively (Table 5).

Table 5. Dairy processing wastewaters emission levels to water bodies achievable with the application of BAT (adapted from EPA, 2008).

PARAMETER	EMISSION LEVEL
pH	**6-9**
BOD_5	**20-40 mg L^{-1}** (or > 90% reduction of influent load)
TSS	**50 mg L^{-1}**
COD	**125-250 mg L^{-1}** (or > 75% reduction of influent load)
TOTAL AMMONIA (AS N)	**10 mg L^{-1}**
TN	**5-25 mg L^{-1}** (or > 80% reduction of influent load)
TP	**2-5 mg L^{-1}** (or >80% reduction of influent load)

According to the latest figures published by the Environmental Protection Agency, the overall picture of fresh water quality in Ireland has decreased for the period 2015 – 2017 (EPA, 2018). Current data reported by the EPA have shown evidence of an increase in nitrate and phosphorus level concentrations at some river sites in the country that could lead to eutrophication issues in the future. Eutrophication is caused by excess of nutrients (mainly sourced from agriculture activities and wastewater discharges) in water bodies that results in water quality depletion due to an overgrowth of aquatic plants. Nutrient concentration limits in wastewater streams have been reduced over time in an effort to protect and to improve the quality of the receiving water bodies (EC, 2000; Stanley et al., 2016). Due to changes in legislation and the fact that some receiving water bodies are of poor quality, Irish dairy processing plants have experienced a reduction in their licenced emissions to surface waters (Figure 6). Dairy industries should be prepared for new discharge policies that could challenge them in improving existing wastewater treatment plants or in adopting new technologies to meet discharge licences.

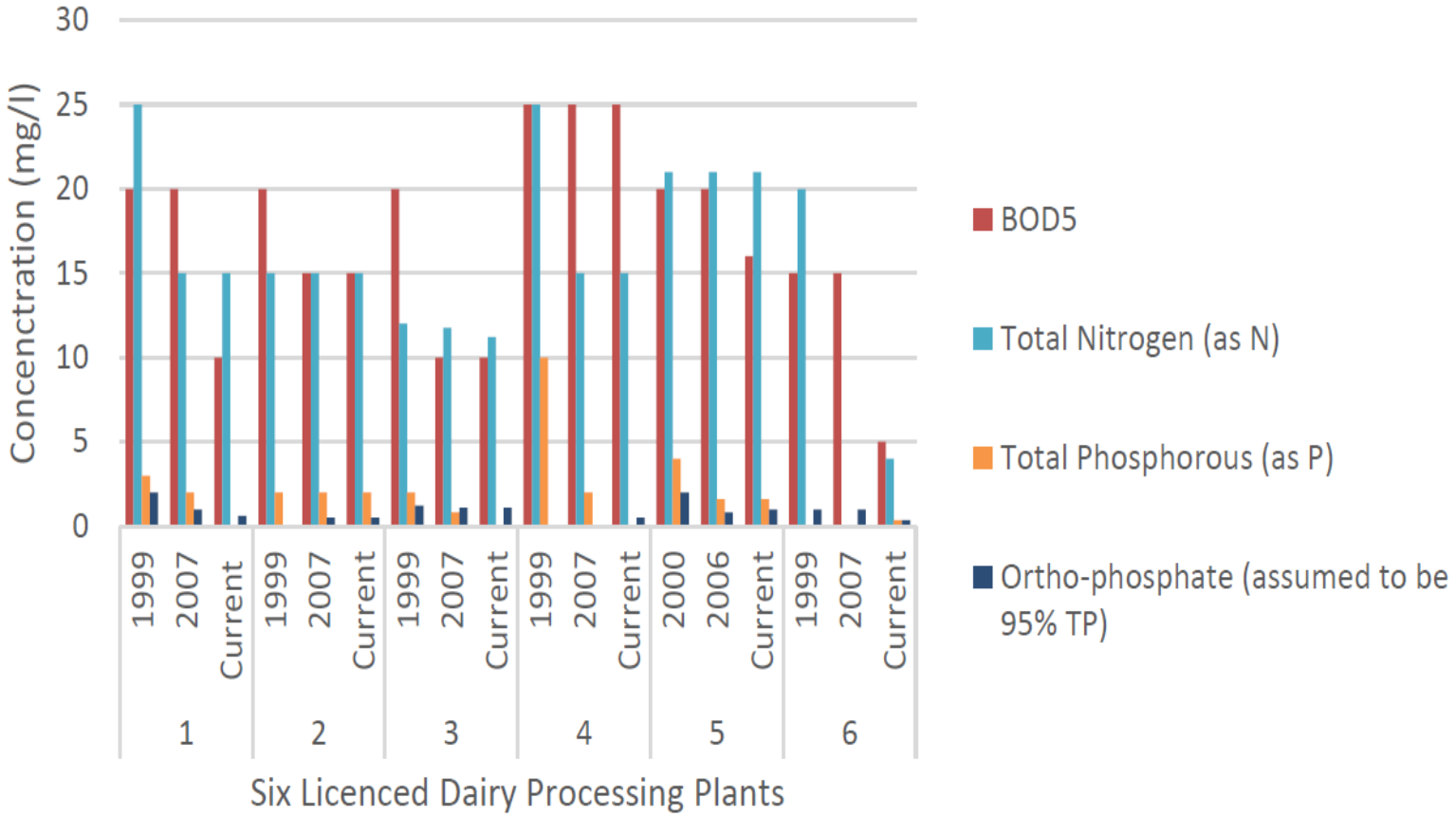

Figure 6. Restrictions in permitted limits for six licenced dairy processing factories in Ireland. BOD_5, Biological Oxygen Demand. (Stanley et al., 2017).

1.5 Treatment of dairy processing wastewaters

Due to the disparities in volume and composition of dairy effluents the selection of an effective wastewater treatment approach can be challenging. There are three main possibilities to treat dairy processing wastewaters prior to discharge: (a) removal of semisolids and special wastes from the site by waste disposal contractors; (b) discharge to nearby municipal wastewater treatment plants; or (c) on-site treatments designed and operated by the dairy facilities (Gough and McGrew, 1993; Robinson T., 1994). On-site treatment is the most common practice adopted in dairy processing facilities prior to effluent discharge into water bodies and typically involves primary, secondary and tertiary processes (Kolev Slavov, 2017; EC, 2006). Primary treatments focus on debris, fat, oil and grease removal and pH balancing (Britz et al., 2006). Extreme pH values are detrimental to the microorganisms responsible for secondary biological treatments and the optimum pH to avoid negative impact in biological processes ranges between pH 6.5 - 8.5 (Eckenfelder, 1989). Secondary treatments are designed for the removal of organic matter and nutrients (N and P) using biological methods and they are recommended for the treatment of dairy wastewaters because of their highly biodegradable nature (Lateef et al., 2013; EPA, 2008). The final step of the on-site dairy processing wastewater treatment involves tertiary processes such as for example chemical precipitation of phosphorus to further improve the effluent quality before it is discharged into surface waters (EPA, 2016).

As outlined in previous sections, the focus on nitrogen and phosphorus removal from dairy processing wastewaters has gained significant attention in recent years. The removal of nutrients from wastewaters can be achieved by using different existing physicochemical and biological processes (Lee et al., 2006; van Loosdrecht et al., 2016). Physicochemical methods such as ammonium air-stripping, breakpoint chlorination and selective ion exchange are used for the removal of ammonium (NH_4^+), the main form of nitrogen in wastewaters (van Loosdrecht et al., 2016). Chemical precipitation of phosphorus has been the main method used for the removal of phosphate (PO_4^{-3}) using metal salts such as ferric chloride ($FeCl_3$) and aluminium

sulphate ($Al_2 (SO_4)_3$) (Stratful et al., 1999, EPA, 2007). In general, chemical precipitation and physicochemical methods of nutrient removal are characterized by high operational costs and result in chemical addition to sludge (EPA, 2007). Biological secondary treatments are an important biotechnological application that consist of aerobic and anaerobic processes, typically in combination, that offer a cost-effective method to remove organic compounds and inorganic nutrients from wastewaters (Wagner et al., 2002; Daims et al., 2006; Bielefeldt, 2009; Lateef et al., 2013; Shete and Shinkar 2013b).

Conventional secondary anaerobic and aerobic biological wastewater treatments are the most common methodologies employed (Britz et al., 2006; Kolev Slavov, 2017). In general terms, both anaerobic and aerobic treatments are suitable to achieve high organic removal efficiencies, but each biological process has its advantages and disadvantages (Demirel et al., 2005; Nadais et al., 2010; Grady et at., 2011). The selection of the most appropriate methodology is based on a balance between technical and economic factors, wastewater characteristics and permitted emission limits. In practice, anaerobic systems have less energy requirements and lower sludge production while aerobic-based processes require high costs of aeration and produce large volumes of sludge biomass (Demirel et al., 2005; Britz et al., 2006; Shete and Shinkar 2013b). However, aerobic processes are less sensitive to temperature changes and generate effluents with better quality in terms of BOD, COD and nutrient removal (Chan et al., 2009; Shete and Shinkar 2013b). Activated sludge processes represents an example of aerobic wastewater treatment, an example of which is illustrated in Figure 7. Anaerobic digestor, anaerobic filters and Upflow Anaerobic Sludge Blanket (UASB), are some examples of typical anaerobic treatments (Figure 8). Disadvantages of anaerobic systems include longer start-up periods for the development of competitive microbial community along with control of the operational temperature for efficient kinetics (Britz et al., 2006).

Biological wastewater treatments are widely adapted to the large variation in dairy effluent compositions with different removal efficiencies achieved (Table 6). In Table 6, bioreactor performances of different biological systems for the treatment of dairy processing wastewaters are summarized.

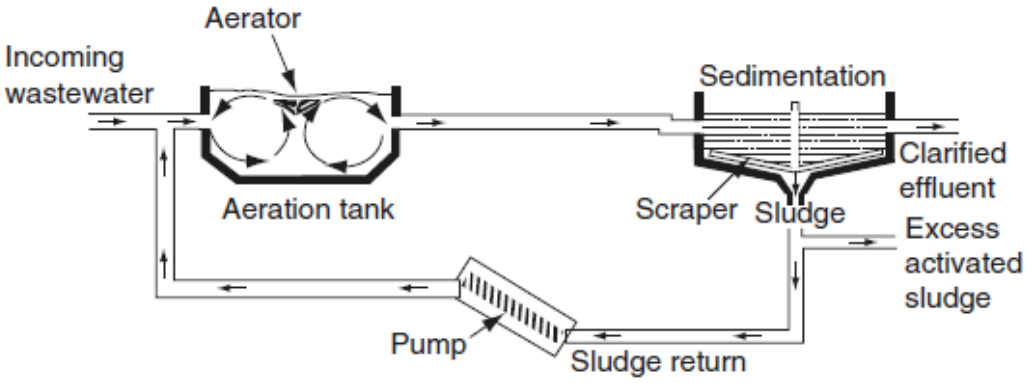

Figure 7. Flow diagram of a conventional aerobic treatment by activated sludge process. (Smith, 2005). When the effluent enters the aeration tank, the wastewater is mixed with the activated microbial sludge. The effluent is aerated by injection of air or oxygen and BOD consumption is promoted in addition to nitrification of ammonium. Then, in the sedimentation tank (or "clarifier") the biological flocs settle separating the clear treated water from the biological sludge, which can be recirculated to the aeration tank.

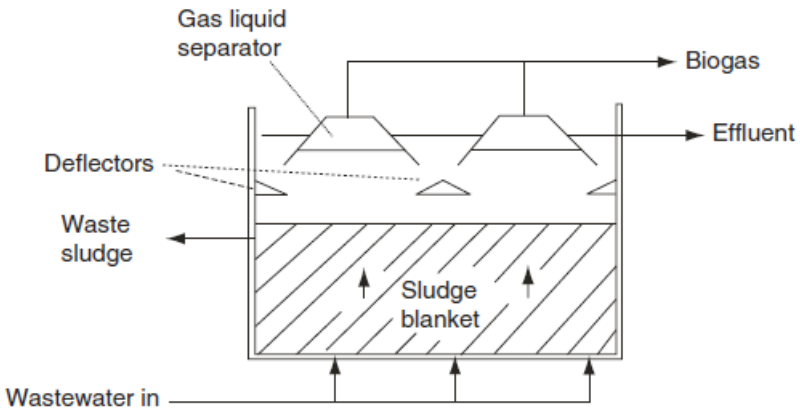

Figure 8. Schematic of an Upflow Anaerobic Sludge Blanket (UASB) reactor for anaerobic wastewater treatment (Smith, 2005). Wastewaters enters the reactor from the bottom and flows upward through the blanket. Then, is processed by anaerobic microorganisms.

Table 6. Performance of different aerobic and anaerobic wastewater treatment systems applied for the biological treatment of dairy processing wastewater. (BOD = Biological Oxygen Demand; COD = Chemical Oxygen Demand; TKN = Total Kjeldahl Nitrogen; TN = Total Nitrogen; TP = Total Phosphorus; SBR = Sequencing Batch Reactor; MSBR = Membrane Sequencing Batch Reactor; UASB = Upflow Anaerobic Sludge Blanket).

System	BOD removal	COD removal	TKN removal	TN removal	TP removal	Reference
Activated sludge	−	>87%	−	97% (as NH_4-N)	−	Sparchez et al., 2015
Activated sludge	−	>90%	−	66%	−	Donkin and Russell, 1997
SBR	−	90%	−	80%	67%	Schwarzenbeck et al., 2005
SBR	~80%	~87%	~49%	−	−	Sirianuntapiboon et al., 2005
SBR	−	80%	75%	38%	−	Li and Zhang, 2002
SBR	90%	70%	−	92% (as NH_3-N)	−	Tam, 1986

System	BOD removal	COD removal	TKN removal	TN removal	TP removal	Reference
MSBR	97-98%	−	96%	−	80% (as PO_4-P)	Bae et al., 2003
MSBR (high organic load)	~83%	~89%	~ 59%	−	−	Sirianuntapiboon et al., 2005
MSBR (low organic load)	~97%	~97%	~79%	−	−	Sirianuntapiboon et al., 2005
Anaerobic filter	−	60-98%	−	−	−	Bonastre and Paris, 1989
UASB	−	90%	−	−	−	Gutiérrez et al., 1991
UASB	−	85-98%	−	−	−	Gavala et al., 1999
ASBR	−	~93%	−	−	−	Sivakumar et al., 2012
DFF	−	90-95%	−	−	−	Cánovas-Díaz and Howell, 1988

In Ireland, the majority of the dairy processing plants use aerobic processes for the treatment of their effluents according to the Environmental Protection Agency (EPA, 2016). Typical aerobic treatments used in Irish dairy processing facilities are Sequencing Batch Reactors (SBRs) and Membrane Sequencing Batch Reactors (MBRs). If dairy wastewater is treated by means of anaerobic processes, facilities have reported their combination with aerobic methods such as bio-towers to achieve better organic and nutrient removals (EPA, 2016). Combinations of biological aerobic and anaerobic methods for the treatment of dairy processing wastewaters have been described previously (Garrido et al., 2001, Sabliy et al., 2009). In a coupled anaerobic filter – sequencing batch reactor system, 99% of nitrogen removal was achieved during the treatment of synthetic dairy processing wastewater that resulted in effluent nitrogen concentrations below 10 mg L^{-1} (Garrido et al., 2001). Additionally, the authors reported that the combination of the methods used in their investigations, anaerobic filters coupled with sequencing batch reactor system, resulted in lower sludge generation and energy consumption. Sabliy and co-workers proposed a combination of two anaerobic bioreactors and three aerobic tanks sequentially connected to treat wastewater from milk plants (Sabliy et al., 2009). The proposed system demonstrated effective removal of COD and Total Nitrogen to ~93% and ~97%, respectively (Sabliy et al., 2009).

Some of the main disadvantages found in the current available treatments for dairy wastewater are related to the use of chemicals, the costs associated to treatment plant operation (EPA, 2016), chemical sludge production and land spreading, non-bioavailable P, etc. This offers a challenging opportunity for the development of new wastewater technologies to be applied in the dairy processing industry to improve existing traditional treatment technologies.

1.6 Biological nutrient removal processes

Biological nutrient removal (BNR) processes are a cost-efficient alternative to physicochemical treatments for the removal of nitrogen and phosphorus based on the activity of different groups of microorganisms (De Lucas et al., 2007; EPA, 2007). In addition, BNR processes offer the possibility of nutrient recovery from wastewater (e.g. phosphorus) and conversion into valuable products such as bioplastics (Bosco and Chiampo, 2010; Yuan et al., 2012). Conventional BNR systems typically required different tanks for the removal of both nitrogen and phosphorus from wastewater (Figure 9). However, sometimes a post-treatment process such as chemical precipitation following the BNR systems is often required to reduce nitrogen and phosphorus levels to meet effluent standards, leading to increases in operational costs. (Yamashita and Yamamoto-Ikemoto, 2014; EPA, 2016).

Biological nitrogen and phosphorus removal is carried out by a core mix of microorganisms with different removal capabilities and growth requirements that contribute together to the nutrient removal processes. At least three different environments (anaerobic, anoxic and aerobic) are needed to enrich for nitrifiers, denitrifiers and polyphosphate-accumulating organisms (PAOs) responsible for nutrient removal within bioreactors (De Lucas et al., 2007; Guo et al., 2013). The stable performance of BNR processes rely on the knowledge and understanding of those microorganism’s communities in order to identify favourable conditions for the successful removal of nutrients and improve process stability and performance. A detailed summary of biological nitrogen and phosphorus removal processes, with focus on known microbiological pathways and key contributors to such processes, is discussed below.

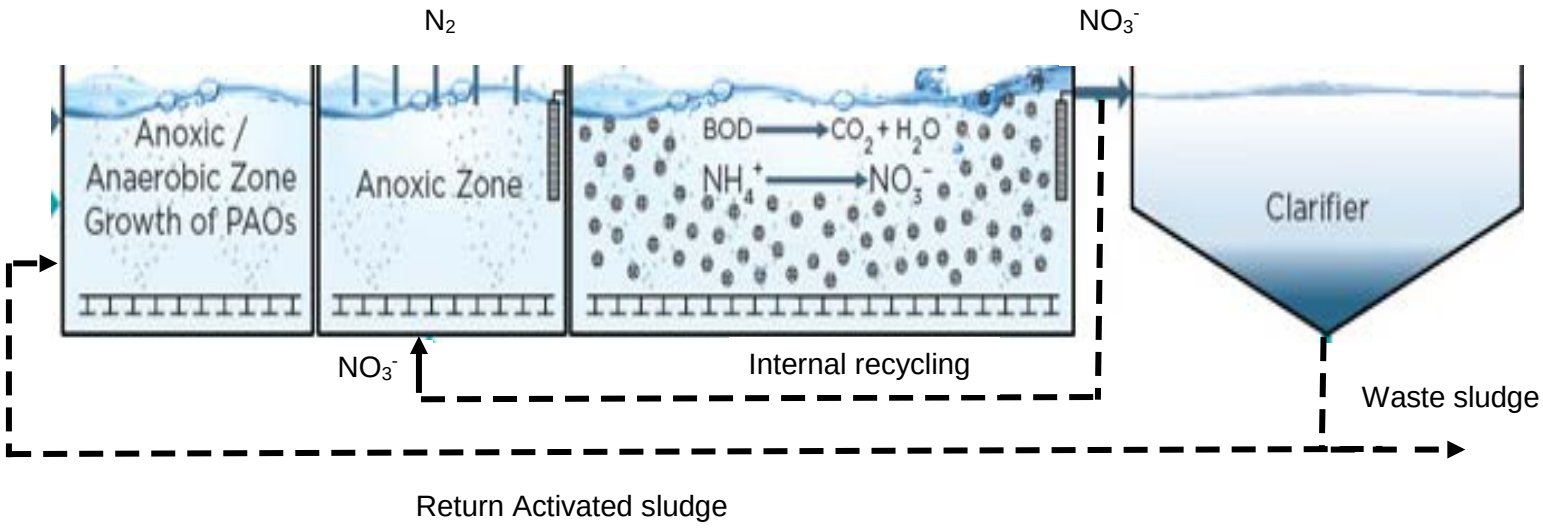

Figure 9. Schematic diagram of a conventional biological nutrient removal system configuration in two stages (Adapted from Headworks International, 2018).

1.6.1 Biological nitrogen removal

Conventional biological nitrogen removal is completed by two-stage treatments composed of aerobic nitrification and anoxic denitrification (Breisha & Winter, 2010). First, ammonium (NH_4^+) is oxidized to nitrite (NO_2^-) and then to nitrate (NO_3^-) by means of different groups of bacteria, namely ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). However, several authors have reported the important role of the domain Archea and their contribution of ammonia oxidizing archaea (AOA) to ammonia removal (Roy et al., 2017; Park et al., 2006) The oxidized form (NO_2^- or NO_3^-) is then reduced to nitrogen gas (N_2O or N_2) by the action of denitrifying organisms (Figure 10).

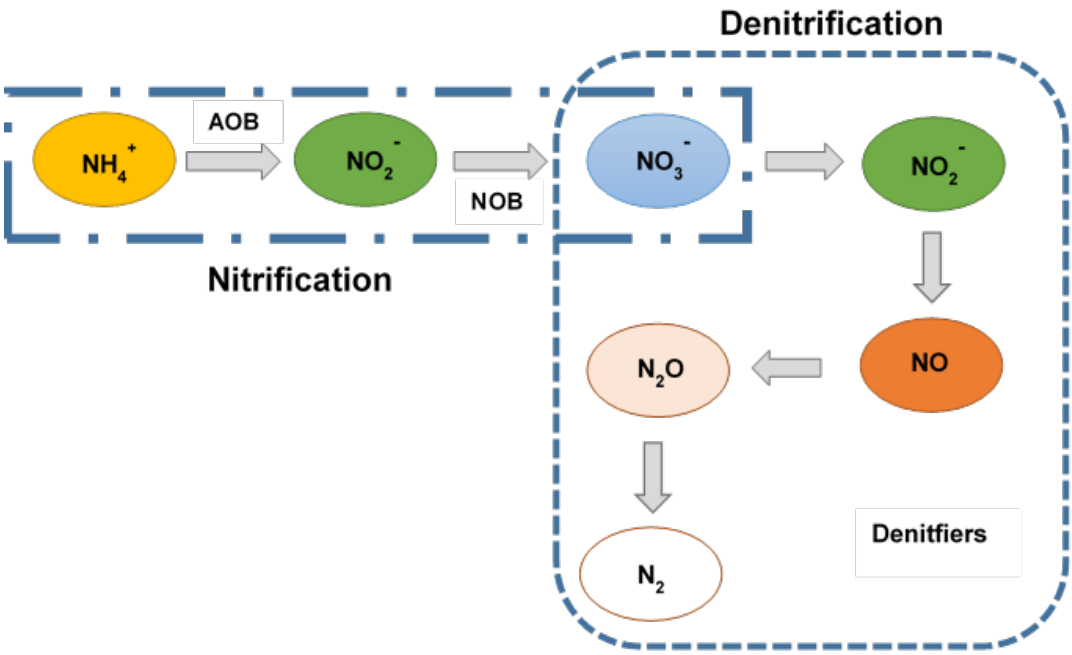

Figure 10. Biological nitrogen removal in two-processes stage: nitrification and denitrification and microorganisms involved.

Nitrification and denitrification reaction pathways are mediated by different key enzymes. Nitrification comprises a three step redox process for the conversion of NH_4^+ to NO_3^- catalysed by ammonia monooxygenase (*amo*), hydroxylamine oxidoreductase (*hao*) and nitrite oxidoreductase (*nxr*,

previously named as *nor*) enzymes (Starkenburg et al., 2006; Ge S et al., 2015; van Loosdrecht et al., 2016) (Eq. 1, Eq. 2, Eq. 3).

$$NH_4^+ + O_2 + H^+ + 2e^- \xrightarrow{amo} NH_2OH + H_2O \qquad \textbf{Eq. 1}$$

$$NH_2OH + H_2O \xrightarrow{hao} NO_2^- + 5H^+ + 4e^- \qquad \textbf{Eq. 2}$$

$$NO_2^- + 0.5O_2 \xrightarrow{nxr} NO_3^- \qquad \textbf{Eq. 3}$$

The overall biochemical pathway for denitrification involves five gene families of denitrification reductases: nitrate reductase (*narG*), periplasmic nitrate reductase (*napA*), nitrite reductase (*nirS*, *nirK*), nitric oxide reductase (*norB*) and nitrous oxide reductase (*nosZ*) (Eq.4). Molecular methods based on functional nitrogen genes have been extensively used to study diversity and community structure of nitrifiers and denitrifiers in biological wastewater treatments (Lu et al., 2014; Fan et al., 2017).

$$NO_3^- \xrightarrow{narG} NO_2^- \xrightarrow{nirS\ nirK} NO \xrightarrow{norB} N_2O \xrightarrow{nosZ} N_2 \qquad \textbf{Eq. 4}$$

Key microorganisms involved in nitrification are known as nitrifiers and comprise two different phylogenetic autotrophic groups of bacteria: ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). Under aerobic conditions, AOB convert ammonia to NO_2^- and NOB subsequently oxidize NO_2^- to NO_3^- in the presence of oxygen (Ge S et al., 2015) (Figure 7). The most highly represented AOBs belong to the genera *Nitrosomonas* and *Nitrosospira* but also other genera such as *Nitrosococcus, Nitrosolobus* and *Nitrosovibrio* (both *Nitrosolubus* and *Nitrosovibrio* not validated taxa to the date of thesis publication) have been detected in ammonia oxidation processes (Nielsen et al., 2009; González-Martínez et al., 2013; Ge S et al., 2015; Ge et al., 2019). With respect to NOB, *Nitrospira*, *Nitrobacter* and *Nitrotoga* sp. have been predominately described as responsible for the oxidation of ammonia to nitrite in biological wastewater systems (Jeyanayagam S., 2005; Huang et al., 2010; Ge S et al., 2015; Lücker et al., 2015). The ability to convert NO_3^- to nitrogen gas in conventional

denitrification is primarily conducted by denitrifying bacteria but it has also been reported in archaea and eukaryotes (fungi) (Lu et al., 2014) (Figure 8). Molecular techniques applied to the study of denitrification communities have indicated the high diversity of organisms capable of transforming nitrite or nitrate into nitrogen gas (Lu et al., 2014; Fan et al., 2017).

The genera *Hyphomicrobium*, *Paracoccus*, *Alcaligenes*, *Bacillus*, *Pseudomonas*, *Azoarcus*, *Zoogloea* and the families *Comamonadaceae* and *Rhodocyclaceae* among others, are commonly found in denitrifying bioreactors (Wu et al., 2013; Lu et al., 2014; Ferrera and Sánchez, 2016).

Molecular based investigations of bioreactor sludge communities and better knowledge of alternative processes for biological nitrogen removal, have allowed the identification of new groups of microorganisms capable of oxidising ammonia using different pathways in the nitrogen cycle such as anaerobic ammonia oxidation (anammox) and complete ammonia oxidizer (comammox) bacteria (Strous et al., 1999; Jetten et al., 2001; Daims et al., 2006, 2015; Ferrera and Sánchez, 2016; Fan et al., 2017). Anammox bacteria belong to the phylum *Planctomycetes* and are capable of oxidising NH_4^+ to N_2O with N_2 as electron acceptor under oxygen absence (Strous et al., 1999; van Loosdrecht et al., 2016). The occurrence of anammox bacteria in laboratory and full scale bioreactors during the removal of nitrogen has been widely studied since the first discovery of ANAMMOX processes in the early 1990s (Innerebner et al., 2007; Kuenen, 2008; González-Martínez et al., 2015). Comammox bacteria have recently been described as part of the microbial structure of wastewater treatment plants which can convert NH_4^+ to NO_3^-, a two-step process in conventional nitrification and carried out by separate groups of microorganisms (González-Martínez et al., 2016b; Fan et al., 2017).

Various, novel biological nitrogen removal technologies based on the metabolic activity of nitrifiers, denitrifiers, anammox and comammox groups of bacteria, have been described as an alternative to conventional nitrogen removal processes (Jetten et al., 1997; Peng & Zhu, 2006; González-Martínez et al., 2015, 2016a). These novel processes include: completely autotrophic nitrogen removal over nitrite (CANON) (Sliekers et al., 2002),

anaerobic ammonium oxidation (ANAMMOX) (Jetten et al., 1998), single reactor system for high activity ammonium removal over nitrite (SHARON) (Dongen et al., 2001), oxygen-limited autotrophic nitrification-denitrification (OLAND) (Pynaert et al., 2004) and partial nitrification-denitrification (Kornaros et al., 2010). The capacity of these systems to improve sustainability and reduce costs when compared to traditional nitrogen removal processes is reflected in a series of advantages such as lower oxygen requirements, less sludge production and no COD requirements (Shalini & Joshep, 2012). For example, coupled partial nitrification and denitrification systems have been shown to reduce aeration costs by 25% and biomass generation by 30% (Gut et al., 2007; Rodríguez-Sánchez et al., 2014). Regardless of the application, the process performance relies on the exploitation of distinct microbial groups involved. The abundance of key bacterial groups is subjected to operational parameters that select for a unique bacteria community structure, influenced by additional environmental factors such as pH and temperature (Ibarbalz et al., 2013; Lu et al., 2014). In addition, it is generally accepted also that bacterial community structure and diversity influence plant stability and robustness of the wastewater treatment (Wagner et al., 2002). The above highlights the importance of conducting investigations to determine the ecological underpinnings of reactor performances for the development and optimization of biological nutrient removal technologies.

1.6.2 Enhanced Biological Phosphorus removal (EBPR)

Biological phosphorus removal (BPR) from wastewaters is achieved by a well-understood and widely described process known as Enhanced Biological Phosphorus Removal (EBPR) (Barnard and Cut, 1974; van Loosdrecht et al., 1997; Mino et al., 1998; Blackall et al., 2002; van Loosdrecht et al., 2016). EBPR represents an alternative to chemical precipitation of phosphorus with high P-removal efficiency, lower operational costs and the potential of nutrient recovery (Janssen et al., 2002; Seviour and Mcllroy, 2003; EPA, 2007; Yuan et al., 2012). In an EBPR process, phosphorus is removed under alternating anaerobic and aerobic/anoxic

cycles by a key group of microorganisms known as Polyphosphate Accumulating Organisms (PAOs). Depending on the oxygen conditions of the environment (aerobic/anoxic and anaerobic stages), PAOs are capable to accumulate intracellular polyphosphate (polyP) and to release stored phosphate (Figure 11).

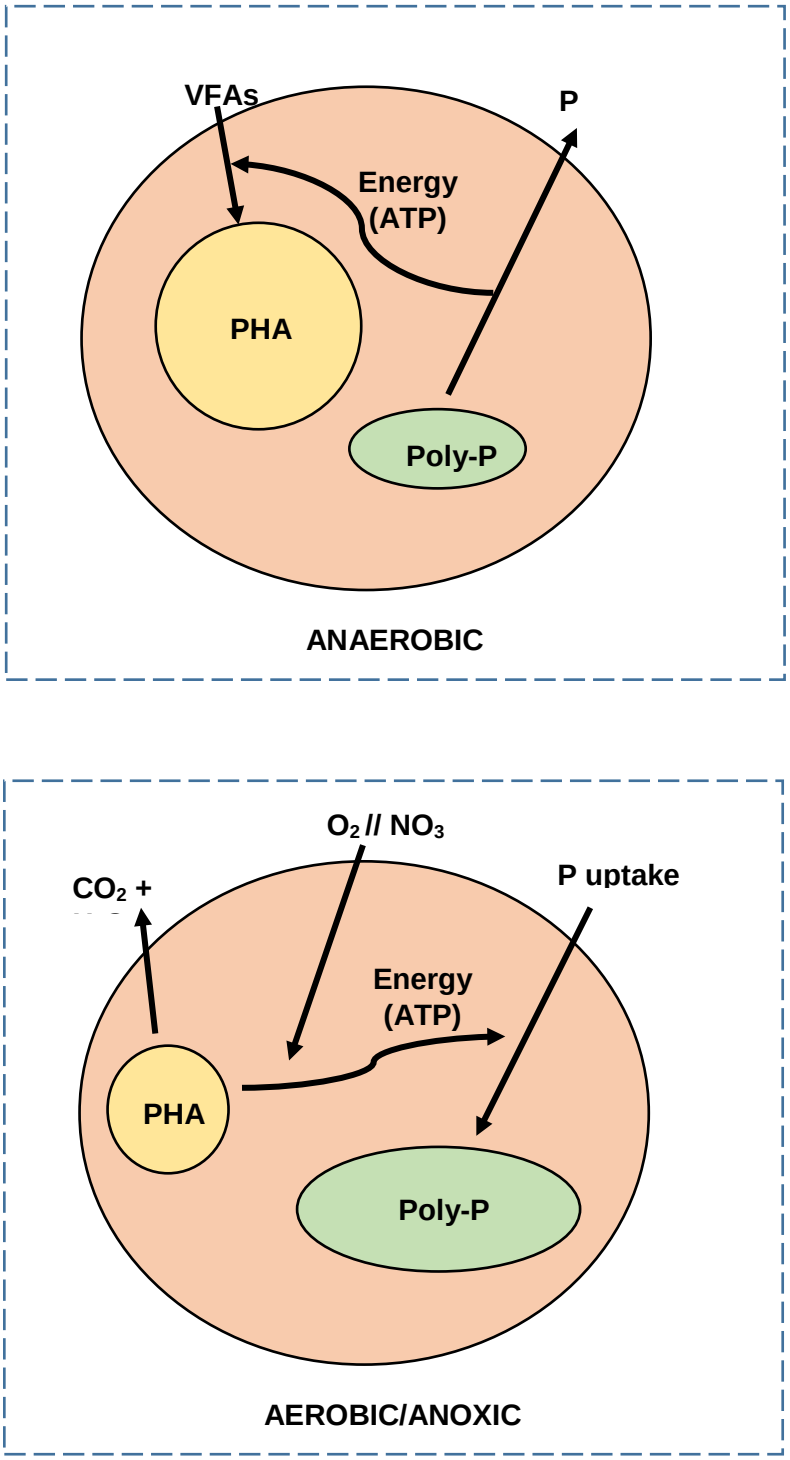

Figure 11. Scheme of biological phosphorus removal mechanism performed by PAOs (Adapted from Jeyanaygam, 2005).

During the anaerobic phase, PAOs store ready biodegradable organic matter (rbCOD) present in the influent (preferably volatile fatty acids -VFAs) as poly-β-hydroxyalkanoates (PHAs). VFAs are used to replenish the cell's stored PHA for subsequent utilisation in the aerobic zone but they can't be used for cell growth during the anaerobic phase (Jeyanayagam, 2005). The required energy for PHA accumulation and transport through the cell membrane is provided mostly from the hydrolysis of polyP and partly from glycogen utilization (Mino et al., 1998; López-Vázquez, 2009). As a result, PO_4^{-3} is released which results in an increase of phosphorus during the anaerobic phase (Mino et al., 1998; López-Vázquez, 2009; van Loosdrecht et al., 2016) (Figure 11). Subsequently, in the aerobic or anoxic zone the stored PHA is used as a carbon and energy source to re-sequester phosphate released during the anaerobic phase and any additional phosphate present in the wastewater to recover the intracellular polyP levels (Jeyanayagam, 2005; McCullagh, 2013). This results in the net removal of orthophosphate from the influent. PAOs also use PHAs to grow, to cover the aerobic maintenance energy needs of PAOs and to replenish the intracellular glycogen pool (Smolders et al., 1994; van Loosdrecht et al., 2016). Net phosphorus removal is accomplished by wasting sludge at the end of the aerobic phase when the sludge contains high levels of polyP (McCullagh, 2013; van Loosdrecht et al., 2016). Glycogen-accumulating organisms (GAOs) are a group of microorganisms detected in EBPR systems with similar metabolism to that of PAOs which do not contribute to phosphorus removal (Erdal et al., 2003; López-Vázquez, 2009). In fact, GAOs outcompete PAOs under certain conditions of temperature, pH and dissolved oxygen (DO) and their presence is often associated with poor EBPR performance (Saunders et al., 2003; Mulkerrins et al., 2004; Oehmen et al., 2007; van Loosdrecht et al., 2016). Several studies have reported the presence of GAOs as the main cause of deterioration of biological phosphorus removal in bioreactors though the mechanisms that influence their occurrence in EBPR systems are still unclear (Oehmen et al., 2007; López-Vázquez, 2009; Kamika et al., 2014). Culture-independent methods based on the study of the 16S rRNA have allowed the identification of key PAOs in laboratory and full scale EPBR reactors. The most important PAOs described to date include *Candidatus* Accumulibacter phosphatis (Crocetti et al., 2000; He and McMahon, 2011)

and genus *Tetrasphaera* (Kristiansen et al., 2013; Marques et al., 2017). However, the potential of other bacterial groups such as *Comamonadaceae* related members and *Rhodocyclus* related organisms in BPR processes has recently been investigated (Zilles et al., 2002; Ge H. et al., 2015). In addition, several families such as *Xanthomonadaceae*, *Saprospiraceae*, *Flavobacteriaceae*, *Cytophagaceae* and *Rhodobactereaceae* have previously been reported to be involved in phosphorus remediation processes in different bioreactor configurations (Kong et al., 2007; Kamika et al., 2014; Valverde-Perez et al., 2016; Xin et al., 2016; Gao et al., 2016). These findings show the need for further research focused on EBPR systems for a better understanding of the microbial ecology underpinning EBRP processes. Indeed, an additional group of microorganisms termed denitrifying phosphorus-accumulating organisms (DPAOs) have recently been shown to exhibit metabolic characteristics similar to those of PAOs involved in BPR (Tsuneda et al., 2006; López-Vázquez et al., 2008,2009; Sun et al., 2015). DPAOs are capable of accumulating polyphosphate by using nitrate (NO_3^-) as an electron acceptor instead of oxygen and the organic carbon substrate can be used simultaneously for both phosphorus and nitrogen removal (Lee et al., 2001; Sun et al., 2015). In recent years, different genera of DPAOs have been described and isolated such as *Comamonas* (Qiang and Han, 2009), *Planctomycetes* (Liu et al., 2013), *Pseudomonas* (Atkinson et al., 2001; Cai et al., 2010) and *Thauera* (Sun et al., 2015). The application of DPAOs provides the opportunity to design new types of wastewater treatment processes for the simultaneous removal of N and P and to solve the problem for organic carbon source competition between denitrifiers and PAOs (van Loosdrecht et al., 1997; Yang et al., 2010; Ma et al., 2013). The benefits and importance of DPAOs in biological systems has been widely recognized in different studies and by various authors in laboratory and full scale investigations (Kuba et al., 1996; Shi and Lee, 2006: López-Vázquez et al., 2008; Qiang and Han, 2009; Cai et al., 2010).

While BPR is an effective remediation strategy, it is sensitive to system disturbances and is dependent on the enrichment of PAOs within the reactor. Therefore, the efficiency of EBPR systems is directly related to the presence of PAOs capable of storing large amounts of polyphosphate (Marais et al.,

1983; Gu et al., 2008; Carvalheira et al., 2014). EBPR process reliability is affected by different parameters such as VFA content, temperature, nitrate sources and solid retention time (SRT) that favour the occurrence of PAOs and minimize the competition with other organisms present in the system (Janssen et al., 2002; Blackall et al., 2002; Mulkerrins et al., 2004; Whang and Park, 2006; Neethling et al., 2006). Conflicting results have been reported in regard to the effect of temperature in EBPR performance. Various studies have concluded that temperatures over 20°C favoured the presence of GAOs over PAOs resulting in a deterioration of phosphorus removal efficiency and that better BPR is achieved at lower temperatures (Whang and Park, 2002; Erdal et al., 2003; Whang and Park, 2006; Keating et al., 2016). However, high phosphorus removal efficiencies have been also reported in SBR systems operating at temperatures ranged from 24 to 32 °C (Freitas et al., 2009; Ong et al., 2014). The integrity of the anaerobic zone and the inhibitory effect of NO_2^- and nitrate NO_3^- have been also examined and associated with the deterioration of phosphorus removal activity in EBPR during anoxic P uptake (van Niel et al., 1998; Ahn et al., 2001; Saito et al., 2004). Results concluded that the anaerobic zone should be protected from nitrate and nitrite sources which position PAOs at a competitive disadvantage with other heterotrophic organisms (Jeyanayagam, 2005). Despite extensive investigations on EBPR systems conducted to date at both laboratory and full scale, the mechanisms affecting relevant microbial communities involved in BPR are still under investigation. In recent years the understanding of key factors controlling the bacterial community structure of bioreactors has evolved, providing insights for new system designs targeting effective management of system ecology and performance.

1.7 Intermittently Aerated Sequencing Batch Reactor (IASBR) technology

Significant commercial opportunities exist for the development of cost-effective, sustainable biotechnologies capable of minimizing the impact of dairy processing wastewater into receiving water bodies. Intermittently aerated sequencing batch reactors, IASBRs, are a biological wastewater treatment based on activate sludge processes but differ from conventional BNR systems in that nitrogen and phosphorus removals are achieved in a single reactor, eliminating the costs associated to traditional multistage treatments, reducing sludge production and removal of precipitants (Orhon et al., 2005; Zhan et al., 2009; Li et al., 2011; Pan et al., 2015). IASBR technology has been developed as an enhancement to the conventional SBR design where multiple, alternating anaerobic periods are imposed during the react phase (Figure 12).

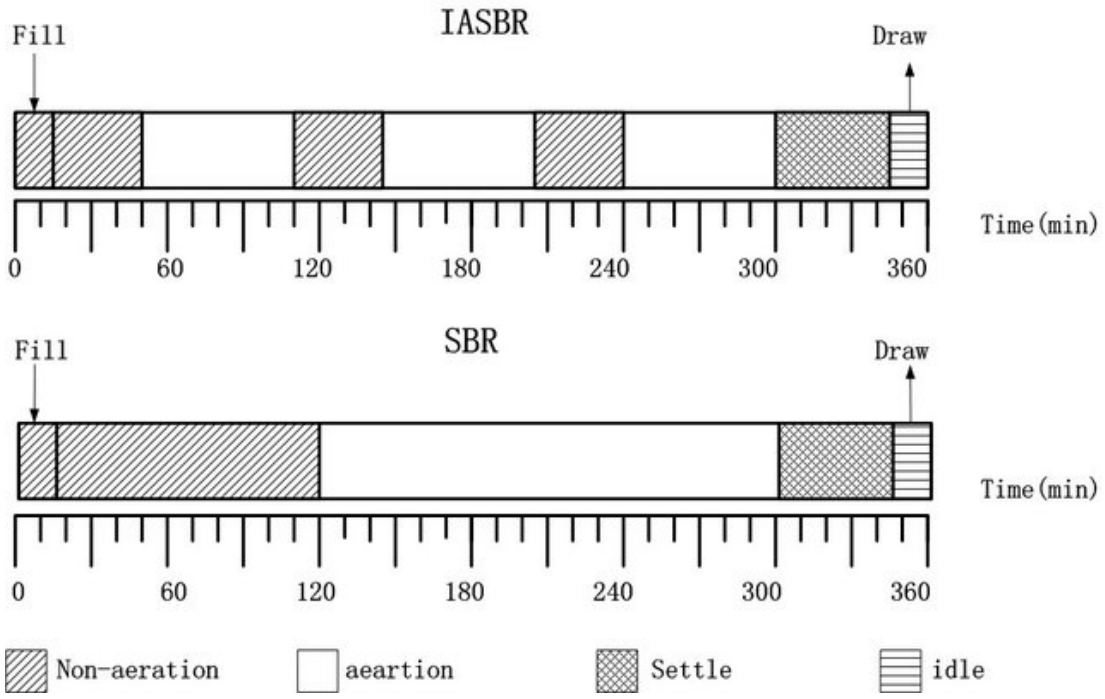

Figure 12. Comparison between operational cycles in Sequencing Batch Reactors (SBRs) and Intermittently Aerated Sequencing Batch Reactors (IASBRs) (Pan et al., 2013).

One complete operational cycle in an IASBR comprises four phases – fill, react (alternating aeration and mixing), settle and draw (Li et al., 2008a). The intermittent aeration strategy applied in IASBR has been shown to achieve long-term, stable partial nitrification resulting in a reduced oxygen demand for ammonia conversion and a reduced organic substrate requirement for subsequent denitrification (Li et al., 2011). Intermittent aeration in SBRs has been successfully applied for the bioremediation of wastewaters of different origins and it has been reported to lead to more stable BNR compared to conventional SBRs (Zhao et al., 1999; Xia et al., 2002; Otawa et al., 2006; Li et al., 2008a, 2008b; Uygur, 2006; Zhan et al., 2009; Li et al., 2011; Rodríguez et al., 2011; Pan et al., 2013, 2014; Henry, 2014; Tarpey, 2016). Pan et al. (2013) compared SBR and IASBR system efficiencies for the removal of nitrogen and phosphorus in synthetic domestic wastewater using SBR and IASBR systems. Considering similar ammonium nitrogen influent in both systems, the total nitrogen (TN) and total phosphorus (TP) removal efficiencies were 79% and 63% in the SBR compared with the 90% and 74% with the application of the IASBR approach. In addition, the simultaneous nitrification and nitrification (SND) efficiency was measured as 90.4% in the IASBR and 79% in the SBR, respectively. Li and co-workers (2008a), reported in their investigations high TN and TP removals from slaughterhouse wastewaters using IASBR technology. An average TN and TP removal of 96% and 99% respectively, were achieved. Those previous investigations have showed the potential of IASBR technology to provide a high efficiency treatment approach for dairy processing wastewater.

The intermittent aeration strategy applied in IASBR systems favour the simultaneous nitrification and denitrification and phosphorus removal reducing the demand for readily biodegradable COD (rbCOD) (Zeng et al., 2003; Orhon et al., 2005; Li et al., 2008a; Li et al., 2011). During the aerobic periods, aerobic nitrifiers oxidize NH_4-N to NO_2^- and NO_3^-, and during non-aeration periods denitrification occurs. The balance of the aeration and non-aeration phase lengths is essential to increase the nutrient removal in the system (Pan et al., 2014). In IASBRs, NH_4-N is partially oxidized to NO_2^- and then is reduced to N_2 gas via partial nitrification (“nitritation”) process (She et al., 2016). Therefore, enrichment of AOB over NOB has to be maintained

(Peng and Zhu, 2006). Pan and co-workers (2014) reported that low aeration rates applied during IASBR treatment of slaughterhouse wastewater favoured AOB growth over NOB, indicating the effect of gradient dissolved oxygen (DO) levels during the intermittent aeration strategy on AOB and NOB quantity. Various factors have been reported to affect the efficiency of IASBRs that will directly impact key bacterial communities responsible of nutrient removal such as the fill strategy and the aeration rate (Tsuneda et al., 2006; Guo et al, 2007; Lemaire et al., 2008; Li et al., 2008b; Henry, 2014; Pan et al., 2014, 2015). Each nutrient removing micro-organism requires specific growth conditions that need to be considered in the design and operation of IASBRs in order to achieve high removal efficiencies. A satisfactory balance must be achieved to meet the requirements of functional groups involved in wastewater treatment by means of IASBR technology for optimal removal of both nutrients. To date, the microbial characterisation of IASBR systems has been limited to few studies (Otawa et al., 2006; Pan et al., 2014). Pan and co-workers (2014) determined the relative, spatial abundance of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) via fluorescence *in situ* hybridization (FISH) to demonstrate the partial nitrification efficiency of IASBR technology treating slaughterhouse wastewater (Pan et al., 2014). In another study carried out by Otawa and co-workers, AOB and bacterial communities were evaluated in 8 animal wastewater treatment plants using intermittent aeration SBR process (Otawa et al., 2006). The overall bacterial community structure of IASBR systems remains still unclear. More comprehensive investigations and further understanding of the bacterial community structure underpinning IASBR systems is essential for optimising biological nutrient removal processes during the treatment of wastewater via IASBR.

1.8 Approaches for the study of microbial diversity in wastewater treatment systems

The recent development of molecular tools for the microbial characterization of bioreactors has enabled a more comprehensive understanding of the bacterial community structure and dynamics in wastewater treatment systems (Ge, S. et al., 2015; Ferrera et al., 2016; Guo et al., 2017). Molecular identification of microorganisms is commonly based on the study of whole genomes or selected genes such as 16S ribosomal RNA (rRNA) (Gurdeep and Rajesh, 2011). The study of 16S rRNA has been used extensively in wastewater microbiology because it is highly conserved among realted bacteria. Additionally, some microbial identifications are also based on targeting key functional genes such as those encoding for enzymes involved in nitrogen removal (Lu et al., 2014; van Loosdrecht et al., 2016).

The variety of molecular techniques existing to date, such as next generation sequencing (NGS), denaturing gradient gel electrophoresis (DGGE) and fluorescent *in situ* hybridization (FISH), can be classified depending on their community or individual approach for the study of the whole or partial community in a given sample (Gurdeep and Rajesh, 2011; Rodríguez et al., 2015; Ferrera and Sánchez, 2016) (Figure 13). The most common methods applied in wastewater research comprise the study of all genes in a microbial community and/or the expressed genes (mRNA) (Rodriguez et al., 2015; van Loosdrecht et al., 2016). Metagenomics techniques based solely on the study of whole sample DNA do not require isolation or cultivation of the microorganisms present, thereby allowing for the inclusion of uncultured microorganisms in analyses. Next-generation sequencing (NGS) techniques, e.g. 454-pyrosequencing and Illumina platforms, have transformed the depth and scale of investigations into the composition and functional diversity of wastewater treatment microbes (Hu et al., 2012; Yu and Zhang, 2012; Kamika et al., 2014; Fan et al., 2017).

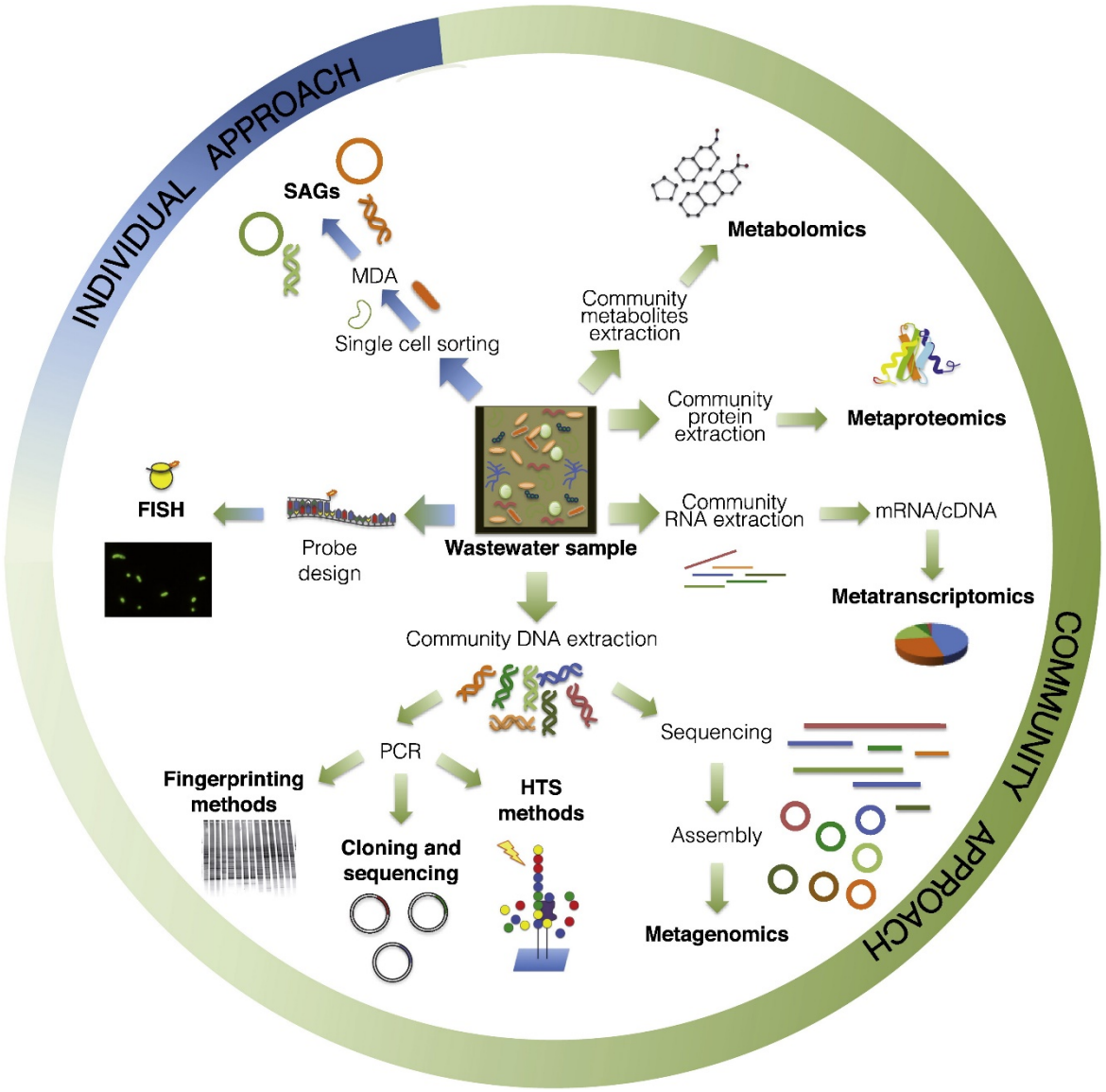

Figure 13. Main molecular tools for the study of microbial ecology diversity and dynamics in wastewater treatment systems (Ferrera and Sánchez, 2016).

As mentioned in section 1.6, molecular-based methods have also allowed investigators to describe and characterize key functional groups involved in nitrogen and phosphorus removal processes (Kamika et al., 2014; Ge H. et al., 2015; González-Martínez et al., 2016b; Guo et al., 2017). Recently, Illumina sequencing has been the most used NGS technology due to the fact that is faster, cheaper and it is more reliable for quantitative assessment of genetic diversity (Lawson et al., 2015; Luo et al., 2012; Guo et al., 2017).

For example, in recent years the discovery of DPAOS (denitrifying phosphorus accumulating organisms) by the use of molecular techniques has provided an opportunity to design new types of wastewater treatment processes for the simultaneous removal of nitrogen and phosphorus with lower sludge production and more efficient use of COD (Tsuneda et al., 2006; Shi and Lee, 2006; Yang et al., 2010). In addition, the complementary use of predictive metagenomic profiling, i.e. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt), has added value to metagenomic data and provided valuable information on functional metabolic capabilities of microorganisms present in wastewater treatment systems (Gao et al., 2016; Guo et al., 2017). Despite the potential of molecular methods, the culture of individual species using culture-depend methods are still important to identify and characterise key functionalities in bioreactor communities (Narayanasamy et al., 2015; Ferrera and Sánchez, 2016). The key to achieve a full-understanding of complex microbial communities associated with biological wastewater treatments relies on integrating metagenomic studies with bioinformatics and statistical tools, which will lead to fully identify microbial communities and their relation to process performance.

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Chapter 2

Evaluation of dairy processing wastewater biotreatment in an IASBR system: aeration rate impacts on performance and microbial ecology

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Abstract

Dairy processing generates large volumes of wastewater that require extensive, on-site nutrient remediation prior to discharge. As a result, significant commercial opportunities exist for the development of cost-effective, sustainable biotechnologies capable of achieving this requirement. In this study the authors evaluated the use of intermittently aerated sequencing batch reactors, (IASBRs), as a single-tank biotreatment system for co-removal of COD, nitrogen and phosphorus from synthetic dairy processing wastewater. Variation of the IASBR aeration rates, (0.8, 0.6 and 0.4 litres/min), was found to have significant impacts on the respective nutrient removal efficiencies and underlying microbial diversity in the IASBRs. Aeration at 0.6 litres/min was most effective and resulted in >90% removal efficiencies for both orthophosphate and ammonium. 16S rRNA based pyrosequencing of biomass DNA samples revealed the family *Comamonadaceae* was notably enriched (>80% relative abundance) under these conditions. *In silico* predictive metabolic modelling also identified *Comamonadaceae* as the major contributor of several known genes for nitrogen and phosphorus assimilation (*nirK, nosZ, norB, ppK, ppX and phbC*).

2.1 Introduction

The European dairy industry is experiencing a period of rapid growth following the abolition of European milk quotas in 2015, with a 50% increase in Irish milk production predicted by 2020. In Ireland, dairy processors consume an average of 2.3 litres of water per litre of milk processed (Finnegan *et al.,* 2015) but can produce up to 10 litres of effluent depending on the end product (Lateef *et al.,* 2013). Such effluents are considered an important source of potential water pollution due to their high nutrient composition. Total Kjeldahl nitrogen (TKN) concentrations up to 1462 mg L^{-1} and total phosphorus (TP) concentrations of 640 mg L^{-1} have been reported in dairy processing wastewater (Britz *et al.,* 2006). Dairy processing plant

effluent discharges into the environment must not impair the quality of the receiving water bodies and

ensure that Environmental Quality Standards (EQS) are not exceeded. Licensed discharge limits can depend on the sensitivity of the receiving water body but typical dairy processing sector limits are currently: 5-25 mg L^{-1} total nitrogen (TN), 2-5 mg L^{-1} TP and 10 mg L^{-1} total ammonia (NH_4-N) (European Commission, 2008). Thus there are numerous drivers for sustainable waste management strategies in the dairy processing sector.

Dairy wastewaters are highly biodegradable and therefore amenable to biological secondary treatments that consist of aerobic and anaerobic processes, typically in combination. Such biological nutrient removal (BNR) systems offer a cost-effective alternative to chemical treatments for the removal of nitrogen and phosphorus (EPA, 2007). In such systems, conventional nitrogen removal is achieved in a two-stage process composed of aerobic nitrification and anoxic denitrification (Breisha and Winter, 2010). Phosphorus remediation also involves aerobic/anaerobic cycling conditions in a process referred to as enhanced biological phosphorus removal (EBPR) (Seviour et al., 2003). Technologies focused on achieving nutrient removal in parallel with improved sustainability have begun to emerge in recent decades. These novel processes include: completely autotrophic nitrogen removal over nitrite (CANON) (Sliekers et al., 2002), anaerobic ammonium oxidation (ANAMMOX) (Jetten et al., 1998), single reactor system for high activity ammonium removal over nitrite (SHARON) (Dongen et al., 2001), oxygen-limited autotrophic nitrification–denitrification (OLAND) (Pynaert et al., 2004), partial nitrification-denitrification (Kornaros et al., 2010) and, simultaneous nitrification-denitrification (SND) and phosphorus removal (Tsuneda et al., 2006). The capacity of these systems to improve sustainability is reflected in advantages such as reduced energy/chemical additive inputs and reduced volumes of sludge biomass and/or chemical precipitants requiring downstream treatment/disposal (Breisha and Winter, 2010). For example, coupled partial nitrification and denitrification systems have been shown to reduce aeration costs by 25%, biomass generation by 30% (Gut et al., 2007; Rodriguez-Sanchez et al., 2014) and process CO_2 emissions by 20% (Kornaros et al., 2010; Shalini and Joseph, 2012).

Intermittently aerated sequencing batch reactors (IASBRs) represent one such BNR process with the capacity for co-remediation of nitrogen and phosphorus within a single bioreactor (Orhon et al., 2005). Each IASBR operational cycle incorporates multiple, alternating anaerobic and aerobic periods, potentially reducing operational costs and sludge production volumes. The intermittent aeration process has been shown to achieve long-term, stable partial nitrification resulting in a reduced oxygen demand for ammonia conversion and a reduced organic substrate requirement for subsequent denitrification (Li et al., 2011). Nutrient removal performances using IASBR technology have previously been assessed for domestic and slaughterhouse wastewater (Li et al., 2008a; Pan et al., 2013a). Pan et al. (2013a) compared SBR and IASBR system efficiencies for the removal of nitrogen and phosphorus in synthetic domestic wastewater. Total nitrogen (TN) and phosphorus (TP) removal efficiencies of 79% and 63% in the SBR system increased to 90% and 74% with the application of the IASBR approach, respectively. In addition, SND efficiencies of 90.4% and 79% were reported in the IASBR and SBR systems, respectively. Li et al. (2008b) reported average TN and TP removal efficiencies of 96% and 99%, respectively, from slaughterhouse influents treated in IASBRs.

Characterisation of microbial diversity and ecosystem function are essential to understanding and optimising biological wastewater treatment processes (Sanz and Köchling, 2007). Previous studies have demonstrated the influence of operational conditions and influent compositions on the microbial ecology of bioreactor systems and associated key metabolic activities of nitrification, denitrification and phosphorus accumulation (Valentín-Vargas et al., 2012; Lee et al., 2015; Gonzalez-Martinez et al., 2016). To date, the microbial characterisation of IASBR systems has been limited to a single fluorescence *in situ* hybridization study to determine the relative, spatial abundance of ammonium (12%) and nitrite oxidizing (7%) bacteria within the general (EUB) bacterial community (Pan *et al.* 2013b). The present study investigated the application of an IASBR to the remediation of synthetic dairy processing wastewater with a focus on the impacts of differing aeration rates, (0.4, 0.6, 0.8 L min^{-1}) and characterisation of the associated microbial communities based on pyrosequencing of 16S rRNA gene V5-V9 hypervariable regions.

2.2 Material and methods

2.2.1 Dairy synthetic wastewater

Six Irish dairy processing plants with on-site wastewater treatment facilities were sampled to determine effluent organic matter, nitrogen and phosphorus levels. The average compositions were as follows: chemical oxygen demand (COD) 3513 mg L^{-1}, soluble COD 3307 mg L^{-1}, TN 122.2 mg L^{-1}, TP 51.9 mg L^{-1}, ammonia (NH_4-N) 48.9 mg L^{-1}, orthophosphate (PO_4-P) 25.4 mg L^{-1}. These characteristics were used to model the synthetic wastewater, incorporating a formulation previously reported by Henry (2014). The final composition contained NaOAc 2929 mg L^{-1}, yeast extract 218 mg L^{-1}, dried milk powder 872 mg L^{-1}, NH_4CL 167.3 mg L^{-1}, urea 129.9 mg L^{-1}, Na_2HPO_4 126 mg L^{-1}, $KHCO_3$ 50 mg L^{-1}, $NaHCO_3$ 130 mg L^{-1}, $MgSO_4 \cdot 7H_2O$ 50 mg L^{-1}, $FeSO_4 \cdot 7H_2O$ 10 mg L^{-1}, $MnSO_4 \cdot H_2O$ 2 mg L^{-1} and $CaCl_2 \cdot 6H2O$ mg L^{-1}. The pH of the synthetic wastewater was 7.9.

2.2.2 Laboratory- scale IASBR system set up and operation

Three laboratory-scale IASBR systems were operated at the Environmental Engineering laboratory in the Department of Civil Engineering, National University of Ireland, Galway. Three identical reactors were operated in triplicate, each bioreactor having an eight litre working volume (Fig. 1). The reactors were located in a temperature controlled environment at approximately 11 °C, in order to replicate average annual temperatures in Ireland. The system was initially seeded with return sludge from a municipal wastewater treatment plant, located in Tuam, Co. Galway (Ireland). The seed sludge contained 8000 mg L^{-1} total suspended solids (TSS) and 6200 mg L^{-1} volatile suspended solids (VSS) respectively, with a 5 L volume being used to inoculate reactors. The operational conditions of the IASBR are summarised in Table 1.

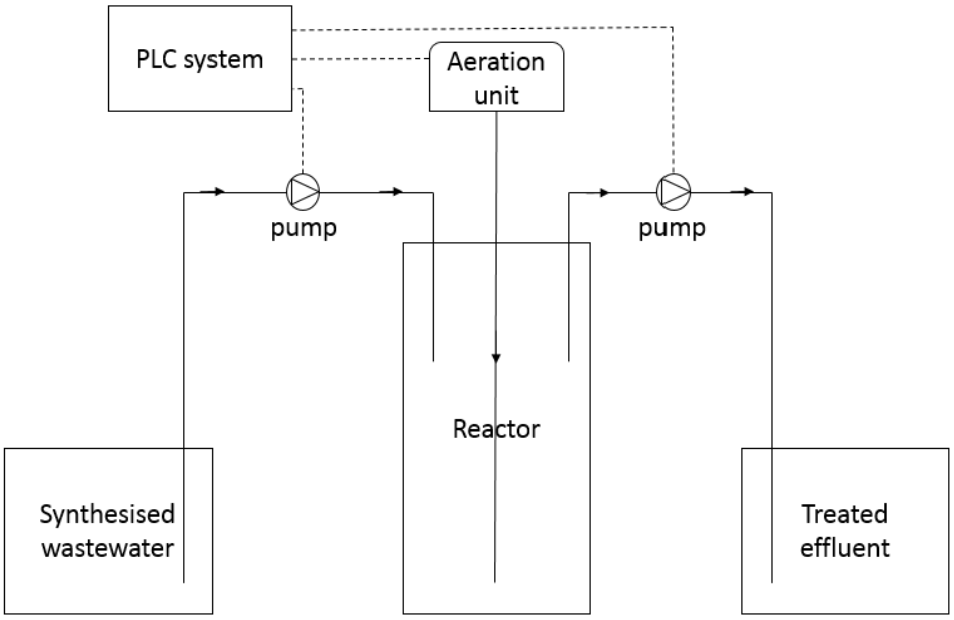

Figure 1. Schematic of the laboratory-scale IASBR system.

Table 1. IASBR operational conditions

Bioreactor volume (L)	8
Hydraulic retention time (days)	4
Solid retention time (days)	20
Temperature (°C)	11
Aeration rate (litres/min)	0.8-0.6-0.4
Operation cycle	
Aeration period (minutes)	60
Non-aeration period (minutes)	100

The IASBR system was operated in 12 hour cycles. At the beginning of each cycle synthetic wastewater was pumped into the system (1 L), followed by four repeat periods of alternating non-aeration (100 min) and aeration (60 min) under continuous mixing. A final 80 min period without aeration or mixing was incorporated to facilitate sludge settling and effluent decanting (800 ml), before the next cycle commenced. A single 400 ml volume of mixed liquor was removed from the reactor once each day as sludge waste, resulting in a 20 day solids retention time (SRT). Samples for metagenomic analyses were collected twice weekly between days 50 to 201. Three different aerations rates were applied during this period: 0.8, 0.6 and 0.4 litre/min. The aeration rates were decided according to preliminary tests of the oxygen concentration profiles in the reactor and from previous work described by Pan et al., 2015, and Li et al., 2008a. At day 55 the initial aeration rate of 1 litres/min was reduced to 0.8 litres/min and sustained for 20 days. Between days 76-161, aeration was further reduced to 0.6 litres/min. On day 161 the aeration rate underwent a final reduction to 0.4 litres/min, which was maintained until the conclusion of the trial on day 201.

2.2.3 Physicochemical profile of the IASBR

Standardized analytical procedures (APHA, 2005) were performed to test influent/effluent suspended solids (SS), dissolved oxygen (DO), chemical oxygen demand (COD) and biological oxygen demand (BOD_5). TN, TP and total organic carbon (TOC) were tested using a Biotector TOC, TN, TP Analyser. In addition, TN and TP were also measured using the HACH TNT methods: 100062, 10127 and 8190, respectively. Quantification of ammonium (NH_4-N), nitrite (NO_2-N), total oxidized nitrogen (TON), orthophosphate (PO_4-P) and calcium carbonate ($CaCO_3$) for alkalinity were analysed using a Konelab 20 Nutrient Analyser (Thermo Scientific), in accordance with the manufacturer's instructions. Parameters were analysed on a daily basis.

2.2.4 Biomass collection and metagenomic DNA extraction

Mixed liquor samples were routinely collected during the third aeration period within the IASBR cycle. A subset of these samples were selected for metagenomic analyses and comprised representatives of each SRT, varying nutrient removal performances and the different aeration rates between days 50 to 201, respectively (Table 2). Samples were collected in sterile bottles and immediately placed at -20 °C until microbial diversity studies were performed at University College Cork, Ireland.

To ensure sufficient biomass for optimal nucleic acid extraction, 6ml of sludge was centrifuged for 15 minutes at 5000 r.p.m, before re-suspending pellets in 1 ml of supernatant. A 300 µl volume of the concentrated biomass was then processed using a PowerSoil DNA Isolation Kit (MOBIO Laboratories) for DNA extraction, according to the manufacturer's instructions. Extractions were quantified via spectrophotometry using a NanoDrop (ND-1000, Thermo-Fisher, DE, USA) and visualized via 1% agarose gel electrophoresis, SafeView (NBS Biologicals) staining and UV trans-illumination.

Table 2. IASBR biomass sampling schedule

Sample ID	T1*	T2	T3	T4	T5	T6	T7	T8	T9
Day since starting	39	62	82	108	131	150	168	182	201
Aeration (litres/min)	1	0.8	0.6	0.6	0.6	0.6	0.4	0.4	0.4

*T1 reference sample represents aeration rate applied during bioreactor stabilisation.

2.2.5 Pyrosequencing and processing of 16S rRNA sequence data

Universal primers U905F (5'-TGAAACTYAAAGGAATTG-3') and U1492R (5'-GGTTACCTTGTTACGACTT-3') with 10 nt unique barcodes (Table S1) were used to amplify the V5-V9 regions of bacterial and archaeal 16S rRNA genes from the extracted DNA (Wang and Qian, 2009; Gao et al., 2015). Each sample was amplified in triplicate to ensure representative sampling. PCR cycling parameters were as follows: initial denaturation at 98° C x 5 min and 30 cycles of 94° C x 40 s, 55° C x 40 s and 72° C x 50 s with a final extension at 72° C for 5 min. PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN) and quantified on a Qubit™ 3.0 Fluorometer (ThermoFisher). The purified products were pooled in equimolar quantities and forwarded to an external service provider for emulsion PCR and 454 GS FLX+ pyrosequencing, MACROGEN (Seoul).

Pyrosequenced amplicon data were corrected using Acacia (Bragg et al., 2012) and subsequent analyses were carried out using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). Chimeras were filtered out and removed using USEARCH v6.1 (Edgar 2010). Quality-filtered sequences (minimum length 200 bp, with no ambiguous bases and homopolymers of 6 bp as maximum) were aligned via PynAST against the SILVA 123 reference database (Quast et al., 2012). Taxonomy was assigned to each OTU using the RDP classifier at a 0.97 threshold. The filtered alignments were clustered into OTUs at the 97% identity level in an open-reference OTU picking process implemented in QIIME.

To compute the diversity analysis, singletons were filtered out from the OTU table before normalizing to ensure that the observed differences were caused by biological origin and not due to random variations in relative sequencing depths (McMurdie and Holmes, 2014). The technique used for normalization was cumulative sum scaling (CSS) (Paulson et al., 2013). Alpha diversity within each sample was calculated following QIIME pipeline procedures.

2.2.6 Predictive functional metabolic modelling

Based on the 16S rRNA sequences, the functional potential of the microbial communities in the bioreactor was predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) approach (Langille et al., 2013). The recommended parameters according to the PICRUSt manual were applied for closed reference OTU picking using the GreenGenes 13_5 reference dataset in QIIME. The OTU table was then filtered for singletons and normalized using the CSS method in QIIME. Using PICRUSt in the web-based Galaxy platform (http://huttenhower.sph.harvard.edu/galaxy), the CSS normalized OTU table was then normalized by known/predicted 16S copy number abundance. Based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (Ogata et al., 2000), the metagenome functional prediction was performed and categorized by the KEGG Orthology (KO) hierarchical levels 1, 3, and 4. In accordance with the PICRUSt guidelines, the Nearest Sequenced Taxon Index (NSTI) cut-off of < 0.15 was used for quality control of the predictions from the samples. Metagenome contributions were computed in PICRUSt for the prediction of the top contributors for target genes of interest. Principal component analysis (PCA) for the functional predictions from the different samples was performed with the vegan package in R, using the RStudio integrated development environment (Team R, 2015). The plots were generated using R built-in functions combined with the ggplot2 package (Wickham, 2009).

2.2.7 Sequence data accession number

Raw sequence data were submitted to the European Nucleotide Archive (ENA, https://www.ebi.ac.uk/ena) under accession no. **PRJEB23305**.

2.3 Results

2.3.1 Nutrient removal performance in the IASBR

Orthophosphate (PO_4-P) and ammonia (NH_4-N) percent removal efficiencies were found to vary depending on the IASBR aeration rate applied, (0.8, 0.6 or 0.4 litres/min), as shown in Figure 2. Sustained nitrogen removal of ~96% was observed for the 0.8 litres/min rate with a concomitant 68% removal of PO_4-P. Under 0.6 litres/min aeration, removal efficiencies of approximately 92% were achieved for both PO_4-P and NH_4-N. The IASBR performance deteriorated following a shift to 0.4 litres/min aeration, with average removal efficiencies of 79% and 57% observed for NH_4-N and PO_4-P, respectively. The capacity of the system to treat synthetic dairy processing wastewater correlated well with previously reported NH_4-N and PO_4-P removal from slaughterhouse effluents subjected to IASBR treatment (Li et al., 2008a; Pan et al., 2013b and 2015.).

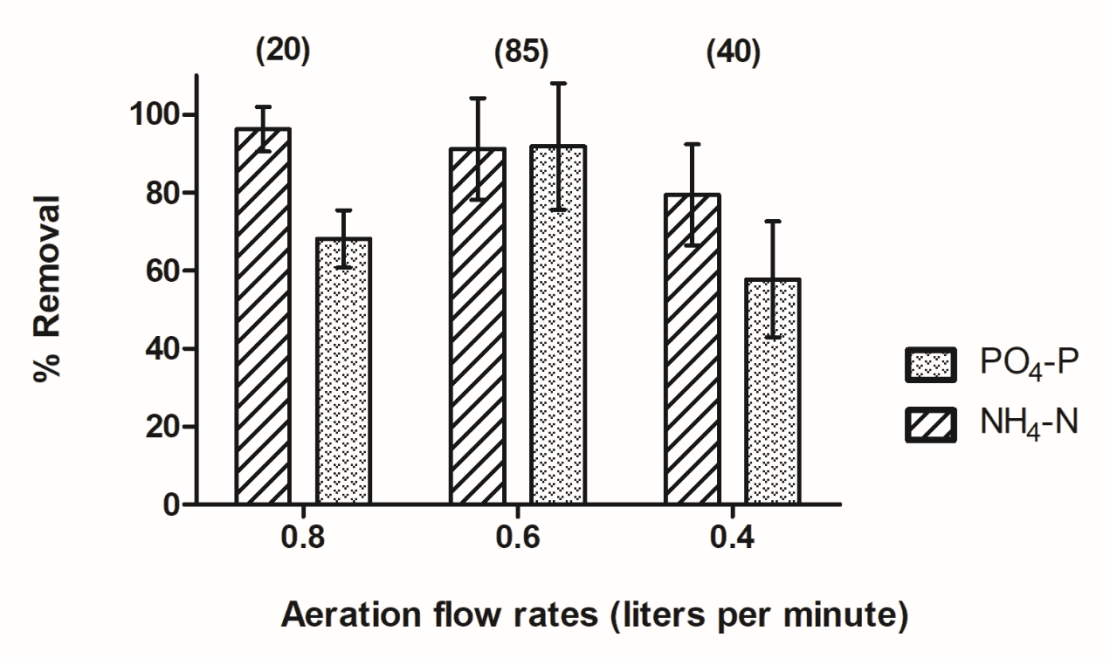

Figure 2. Average % removal of PO_4-P and NH_4-N at varying aeration rates. In brackets, the duration of each aeration period in days.

2.3.2 System community richness

A total of 82,176 high quality reads were detected after bioinformatics quality control analyses. The coverage index for each sample was ≥0.8, suggesting that the relative number of species were well represented among samples, (Table 3). With respect to alpha diversity metrics, the library size of each sample was normalized due to varying depths of coverage across the samples. The species richness, calculated by Chao 1 index and the observed OTUs at a 3% cut off level, is summarized in Table 3.

2.3.3 Microbial ecology profiling of the bioreactor

Bacterial community compositions in the IASBR system were determined using the high-throughput pyrosequencing method which targeted the V5-V9 region. Only sequences with OTU assignment similarities of ≥ 97% were included in the analyses. Ecological profiles at family level are shown in Fig. 3. In summary, 12 dominant families, (defined as having ≥1% total relative abundance), were identified. The “Other minor families” category represents grouped families with total relative abundance values lower than 1%. A notable observation was the dominance of the *Comamonadaceae* family within the IASBR community profile and the impact of the relative aeration rates on their overall levels. In the reference sample (T1), the abundance of *Comamonadaceae* was 18.8% and increased up to a maximum of 43.7% within the first aeration rate investigated (0.8 litres/min). In the subsequent shift to reactor operation at 0.6 litres/min over 4 SRTs, (T3-T6), *Comamonadaceae* relative abundance steadily increased to sustained maxima of 87% (T5) and 82.1% (T6), respectively. The final reduction in reactor aeration to 0.4 litres/min correlated with a gradual decrease in *Comamonadaceae* from days 168 (T7) to 201 (T9), where levels dropped from 68.9% to comparable reference sample values of 16.3%. While *Comamonadaceae* dominated the majority of profiled samples, other families previously reported to be involved in nitrogen and phosphorus remediation processes were also observed e.g. *Flavobacteriaceae*

and *Rhodocyclaceae* (Guo *et al.,* 2016; Kamika *et al.*, 2014; Kong *et al.,* 2007). However, their low, respective relative abundances of 1.7% and 0.4% during optimal performance under 0.6 litres/min, (see T5 and T6 in figure 3), appears to suggest a limited role in the system.

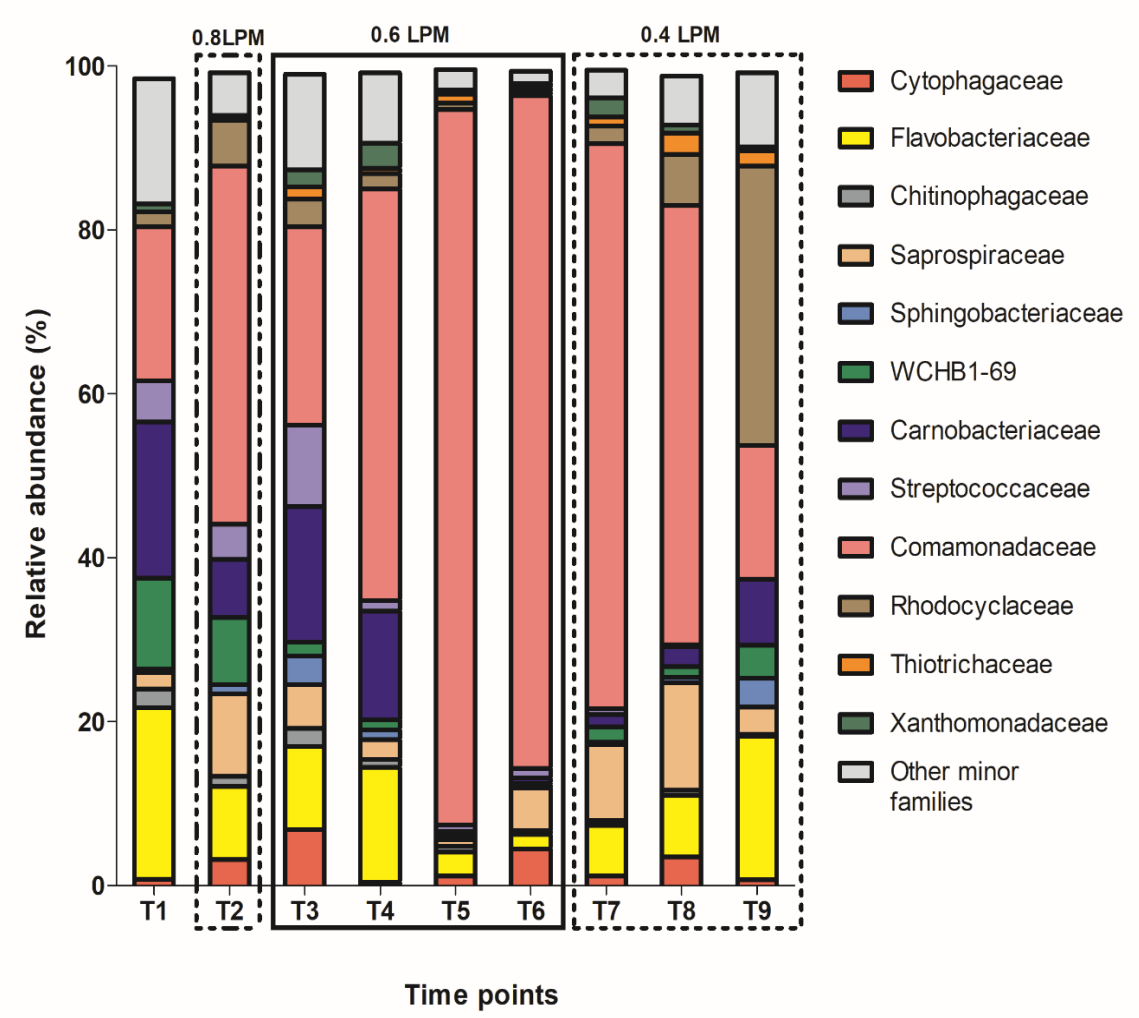

Figure 3. Overview of IASBR bacterial community structure at family level. T1 to T9 represents differing sample time-points (see Table 2).

Table 3. Diversity and species richness within the metagenomic dataset

Sample ID	T1	T2	T3	T4	T5	T6	T7	T8	T9
High quality reads	11675	13220	5568	9994	13770	10182	4936	5026	7805
Normalized reads	1914	2047	1495	1703	1290	1199	1072	1144	1462
Observed OTUs	674	676	493	551	366	355	314	329	416
Chao 1 index	740.5	789	719.8	776.5	366	355	314	329	416
Coverage index	0.9	0.9	0.8	0.8	1	1	1	1	1

2.3.4 Functional potential of the microbial communities

PICRUSt predicted metabolic functionality from the metagenomic profiling of microbial communities in the IASBR at KO hierarchy level 1 is shown in Fig 4. Among predicted KEGG pathways, “Metabolism” (50.14%) was the most abundant category followed by “Genetic Information Processing” (15.98%), “Unclassified” (14.66%), and “Environmental Information Processing” (14.23%). Principal Component Analyses (PCA) were computed to investigate potential correlations between bacterial community metabolic profiles and the varying aeration rates applied to the IASBR system. As shown in Fig. 5, three distinct clusters emerged which indicated a shift in the functional/metabolic profiles of the microbial communities in response to the varied aeration conditions. The plots also revealed the time dependent nature of these shifts, e.g. T3-T4 versus T5-T6 during 0.6 litres/min aeration conditions, which correlate with the observed bacterial diversity profiles shown in Fig. 3. The KO database also facilitated analysis of the metagenomic data set for relative abundances of genes known to contribute to nitrogen and phosphorus remediation. Key genes associated with denitrification (nitrite reductase (*nirK*), nitric oxide reductase (*norB*) and N_2O reductase (*nosZ*), and EBPR processes (polyphosphate kinase (*ppk*), exopolyphosphatase (*ppx*) and polyhydroxyalkanoate synthase (*phaC*) were identified. OTUs contributing the genes of interest described above were then computed using PICRUSt. In order to select the top contributors to the genes of interest, OTUs for the metagenome prediction were merged up to the family level. Taxa that did not contribute ≥ 1% of the total relative abundance for one or more of the genes of interest were excluded. As shown in tables 4 and S1, *Comamonadaceae* represented the top contributor for the described genes, which correlated with their observed taxonomic dominance in the IASBR system (Fig. 3). However, it was also noted that some of the less well represented taxa, such as for example *Xanthomonadaceae* (2.2% relative abundance), had a significant contribution [1]to the predicted functional profile of the microbial communities (Table S1).

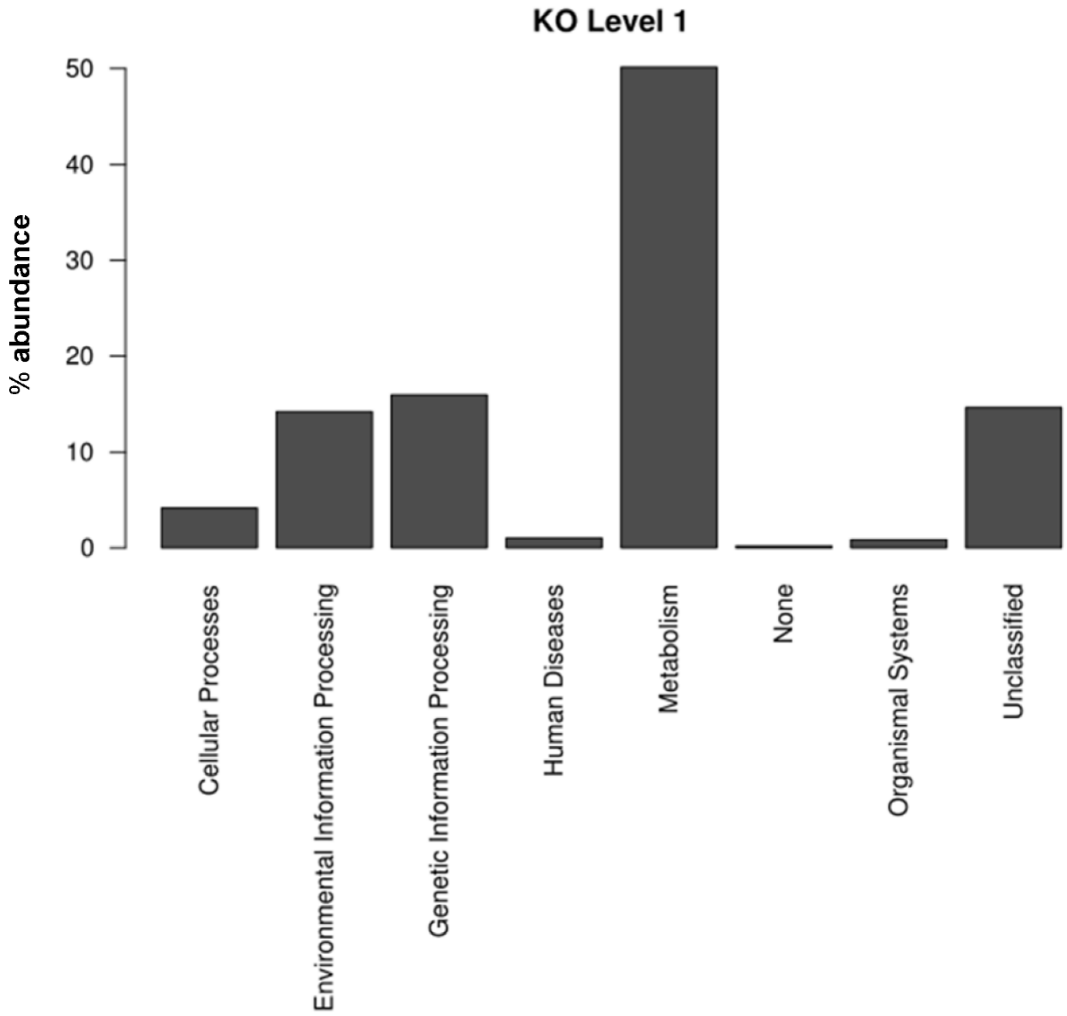

Figure 4. Functional predictions of bacterial diveristy of IASBR treating dariy synthetic wastewater. KEGG metagenome functional predictions of OTUs at KO.

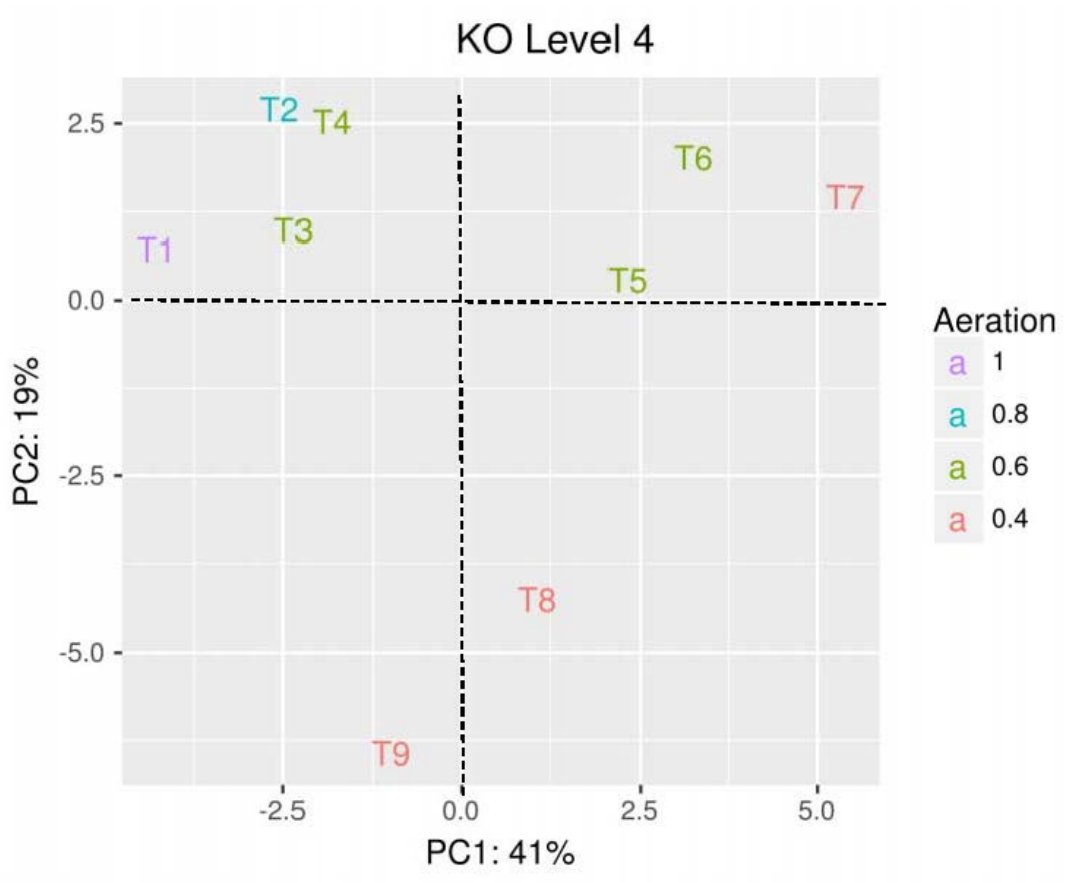

Figure 5. Principal Components Analyses (PCA) at gene level (KO level 4) considering the aeration rates and the time-points.

Table 4. Correlation of taxonomy (up to family level) and relative contributions to genes of interest: *ppk*, *ppx*, *phaC*, *nirK*, *norB* and *nosZ* [a].

Total relative abundance (%)	Taxonomy	Relative gene contributions (%)					
		ppk	***ppx***	***phaC***	***nirK***	***norB***	***nosZ***
38.2	*Comamonadaceae*	30.9	30.3	58.3	5.10	50.1	31.1
10.4	*Order SC-I-84*	0.88	0.88	1.71	-	1.75	-
8.2	*Rhodocyclaceae*	6.19	6.71	13.7	17.3	12.3	22.8
8	*Flavobacteriaceae*	11.6	10.7	-	20.7	8.03	8.27
4.1	*Thiotrichaceae*	3.16	3.14	6.11	-	-	-
3.6	*Saprospiraceae*	6.68	3.15	-	-	-	16.9
2.4	*Cytophagaceae*	2.27	4.31	-	-	-	0.51
2.2	*Xanthomonadaceae*	10.3	10.1	9.90	34.6	15.8	0.71
1.5	*Weeksellaceae*	2.57	2.55	-	9.48	4.04	0.92
0.9	*Chitinophagaceae*	4.44	2.96	-	-	-	7.73
0.6	*Sphingobacteriaceae*	4.44	4.12	-	-	-	-
0.4	*Porphyromonadaceae*	1.03	0.71	-	-	-	-
0.4	*Peptococcaceae*	-	1.30	1.27	-	-	-
0.4	*Rhodobacteraceae*	1.45	2.88	1.41	6.85	2.65	6.53
0.3	Order *Bacteroidales*	1.29	1.14	-	-	-	-
0.3	*SB-1*	1.11	1.10	-	-	-	-
0.3	*Cryomorphaceae*	0.91	1.16	-	-	-	-
0.1	*Lachnospiraceae*	1.24	0.31	-	-	-	-
0.1	*Sinobacteraceae*	0.94	0.94	1.83	-	-	-

"- "= no detected contribution of gene of interest.

[a] 1% cut-off was applied.

2.4 Discussion

2.4.1 Impact of the aeration rates in nutrient removal performance

The dairy industry forms a key pillar of the agri-food sector in numerous countries with projected 2020 global milk production outputs estimated to reach approximately 830 million tonnes (Bojnec and Ferto, 2014). A significant percentage of liquid milk undergoes processing into a range of consumer products such as whey protein, butter, cheese and milk powder, which can consume 2 – 6m^3 of water per tonne of milk processed (Demirel et al., 2005). The resulting high volumes of wastewater can present a considerable remediation challenge due to high nutrient loadings ranging from 3-70 kg/m^3 COD, 0.05-1.4 kg/m^3 total nitrogen and 0.01-0.7kg/m^3 total phosphorous, respectively (Minescu et al., 2016). The potential of IASBR technology for high efficiency nutrient removal from industrial as well as domestic wastewaters has previously been reported (Li et al., 2008b; Pan et al., 2013a; Pan et al., 2013b; Pan et al., 2015). In the current study the scope of IASBR application has been expanded to include the potential remediation of dairy processing wastewater. In summary, optimum PO_4-P and NH_4-N co-remediation efficiencies (>90%) were achieved with synthetic dairy wastewater at 0.6 litres/min, but the IASBR performance was found to be significantly affected at aeration rates above or below this value (i.e. 0.8 or 0.4 litres/min, respectively). When the aeration rate was 0.4 litres/min, the DO concentrations in the aeration periods were low, potentially reducing the ammonium oxidation rate by autotrophic nitrifiers. It may also explain lower phosphorous uptake by phosphorus accumulating organisms (PAOs) wherein polyphosphate accumulation occurs under aerobic conditions in conjunction with intracellular polyhydroxyalkanoate degradation. Overall results appear to suggest that under optimal aeration, IASBR could offer an effective treatment option for dairy processing wastewaters, coupled with reduced energy and infrastructural demands when compared with traditional BNR multistage systems.

2.4.2 IASBR microbial community profiling

It is generally accepted that microbial communities within activated sludge are affected by changes in bioreactor configurations, which can exert influences over system stability and robustness (Wagner and Loy, 2002; Werner et al., 2011). However, IASBR systems are not well characterised in this regard. In an effort to establish some insight into IASBR community structure the authors applied next generation sequencing (NGS) to 16S profiling of multiple samples collected under varying operational aeration rates. Subtle changes in the aeration rates appeared to significantly impact on the observed community structure within the reactor (Fig. 3). The most notable observation was the dominance of the family *Comamonadaceae* within the biomass, (82-87% relative abundance), at 0.6 litres/min aeration; which coincided with optimal nutrient removal performance within the reactor (Fig. 2). It is possible that a threshold oxygen concentration provides a selective pressure for *Comamonadaceae* specific metabolism which becomes optimal under 0.6 litres/min. A partial enrichment appears to operate under 0.8 litres/min. However, the competitive advantage appears to dissipate at 0.4 litres/min and, rather than drop off sharply, *Comamonadaceae* gradually decline toward reference sample levels over a 60-day period. Xin and co-workers recently demonstrated that varying aeration pressures, (0.2-0.6 MPa), significantly impacted on the relative abundance of *Comamonadaceae* in a sequencing batch reactor kettle (SBRK) system treating municipal wastewater (Xin et al. 2016). In an earlier study, Sadaie and colleagues reported the gradual dominance of *Comamonadaceae* (52.3%) following reduced air supply (<1mg/L) to a conventional activated sludge process treating food processing waste (180 m^3, BOD_5 =1000mg/L) (Sadaie *et al.,* 2007). The disparity between the compositions of municipal, food processing and dairy wastewaters suggests the influent is unlikely to be the selective pressure in *Comamonadaceae* enrichment, but rather reduced dissolved oxygen. Several *Comamonadaceae* species, belonging to at least 12 different genera, have been isolated from activated sludge and linked with nutrient removal from wastewaters (Weissbrodt et al., 2014; Willems, 2014; Xin et al., 2016).

Evidence from the literature suggests a positive correlation between several members of the *Comamonadaceae* and denitrification processes (Calderer et al., 2014; Willems, 2014). Recently, Ge and colleagues reported a novel clade within *Comamonadaceae* linked with high capacity phosphorus uptake from abattoir waste streams (Ge et al., 2015). The authors achieved >90% orthophosphate removal, (influent load 24 mg L^{-1}), in an SBR system operated at a solid retention time of <4 days. Fluorescent *in situ* hybridisation (FISH) and intracellular poly-phosphate granule staining with 4',6'-diamidino-2-phenylindole confirmed *Comamonadaceae* representatives as key contributors to orthophosphate uptake within the system. Collectively, these recent studies suggest that *Comamonadaceae* members may well play a number of important roles in biological nutrient removal processes where they constitute a sizeable fraction of the microbial biomass.

2.4.3 Predictive metagenomic profiling of the IASBR microbial community metabolome

In order to gain a fuller understanding or describe the microbial ecology of a system, functional correlations are required. In an effort to fully mine the ngs data for potential correlations between taxanomic abundance and possible contributors to nutrient removal efficiencies, a predictive modelling approach, PICRUSt, was applied. Ahmed et al. (2017) previously employed this approach to model the diversity and abundance of antibiotic resistance genes in raw versus secondary effluents from four Australian municipal treatment facilities. In a separate study, Gao and co-workers also employed PICRUSt analyses to suggest that the removal of pathogenic microorganisms from sewage sludge via anaerobic digestion did not significantly reduce the genetic capacity within the sludge to contribute "human disease" (Gao et al., 2016). Our study represents the first application of PICRUSt modelling on an IASBR system.

IASBR systems have been reported to involve partial nitrification to remove nitrogen via nitrite intermediates (Pan et al., 2013b; Mota et al., 2005). Such processes require aerobic denitrifiers and the associated *nirK*, *norB* and *nosZ*

genes (Wan et al., 2011). PICRUSt analysis indicated that *Comamonadaceae* potentially contribute >50% of the *norB* and >30% of the *nosZ* genes within the community. With respect to phosphorus removal genes, *ppk* and *ppx*, were selected regarding their roles in poly-P synthesis and degradation, respectively (Zheng et al., 2011; Chen et al., 2014). Poly-hydroxy-alkanoate (PHA) metabolism has also been linked with EBPR, and involves a *phaC* encoded synthase (Willems, 2014; Sakai et al., 2015). In our analyses *Comamonadaceae* was also predicted to be the top contributor of *ppk, ppx* and *phaC* genes within the community (Tables 4 and S1). The authors did note that a strict relationship between relative taxonomic abundance and metagenomic contribution was not observed. *Rhodobacteraceae* for example, a known denitrifying proteobacteria (Motlagh and Goel, 2014: Heylen et al., 2006) accounted for only 0.4 % relative taxonomic abundance within the dataset, however its predicted functional contribution of denitrification genes was over 6% for *nirK* and *nosZ* genes (Table 4 and S1). Further, comprehensive analytical investigation of the IASBR system will however be required (e.g. FISH, biopolymer specific staining and gene expression analyses) to establish the functional significance of the modelled outputs and to provide further insights into our understanding of the microbial ecology underpinning successful IASBR application.

2.5 Conclusions

With the introduction of legislation such as the EU Water Framework Directive (Directive 2000/60/EC) and more stringent licensing requirement, cost efficient, sustainable treatment of wastewater is becoming increasingly important. IASBRs have the potential to provide a high efficiency treatment approach for dairy processing wastewater; reducing the need/costs for high level aeration and chemical precipitant addition while decreasing the volume of sludge produced. The single reactor IASBR system also offers a reduced infrastructural footprint when compared with traditional anoxic/oxic multistage systems. In conclusion, IASBR application to dairy processing wastewater

remediation is a promising technological approach. However, optimisation is critically dependent on operational aeration rates, which greatly influence the ecological shifts within the system. Metagenomic based metabolic profiling suggests members of the *Comamonadaceae* family may contribute significantly to nitrogen and phosphate remediation processes. Currently, the authors are investigating functional correlations between the IASBR performance and ecological profiles reported here, in addition to determining the impacts of real-time dairy processing wastewater inputs to the system.

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Chapter 3

Microbial ecology of IASBR laboratory systems treating synthetic and industrial dairy processing wastewaters: Impact of influent characteristics on community dynamics and *Comamonadaceae* dominance.

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Abstract

Due to the potential environmental risk associated with dairy processing wastewaters and increasingly stringent effluent discharge regulations, the removal of organic matter, nitrogen and phosphorus from such wastewaters has gained significant attention in recent years. Intermittently aerated sequencing batch reactors (IASBRs) represent a biological nutrient removal technology combining multiple anaerobic/aerobic operational cycles capable of achieving chemical oxygen demand (COD), nitrogen (N) and phosphorus (P) removal in a single reactor. The study presented here compared microbial community structure and dynamics of two laboratory-scale IASBRs treating synthetic and industrial dairy processing influents. Functional profiling of bacterial families contributing key genes of known phosphorus and nitrogen metabolic pathways was also conducted. The stable dominance of *Comamonadaceae* groups was noted in the synthetic influent system where *Polaromonas* and other *Comamonadaceae* accounted for 11% and 53% average relative abundance, respectively. However, the treatment of industrial dairy processing wastewater was associated with a significantly higher overall bacterial diversity with reduced dominance of *other Comamonadaceae* (9% relative abundance). Statistical analyses by means of RDA suggested that influent characteristics such as varying compositions of NH_4 -N and PO_4 -P had a greater shaping influence on microbial community structure than operational conditions applied in the study. Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) identified *Comamonadaceae* as the key contributor of nitrogen and phosphorus assimilation/storage pathway genes.

3.1 Introduction

Dairy processing can generate large volumes of wastewater per unit of product (Brião and Tamares, 2007; Shete and Shinkar., 2013a; EPA 2016), particularly when frequent cleaning procedures are required where a production unit may have a diverse product portfolio (Shete and Shinkar, 2013b; Rad and Lewis 2014).

Such dairy effluents are characterized by varying compositions with high loadings of nitrogen (N), phosphorus (P) and organic matter (Danalewich et al., 1998; Kushwaha et al., 2011; Britz et al., 2006; Tikariha and Sahu, 2014). Total concentrations of biological oxygen demand (BOD_5) up to 4,790 mg L^{-1} and chemical oxygen demand (COD) concentrations of 500−4,500 mg L^{-1} have been reported in untreated dairy effluents (EA, 2009; EAEW, 2000). In a recent study of 6 Irish dairy processing plants, concentrations for key inorganic nutrients were found to range between 0.9−184.2 mg NH_4-N L^{-1} and 5.0−102 mg PO_4-P L-1 (Finnegan et al., 2018).

Removal of nitrogen and phosphorus from dairy wastewaters has recently gained significant attention due to the potential environmental risk they pose to receiving water bodies (Shete and Shinkar., 2013a; Britz et al., 2006; Fan et al., 2017) and stricter environmental regulations (Stanley et al., 2017). Several studies have highlighted the use of biological nutrient removal (BNR) technologies, in conjunction with other processes, for the successful removal of organic matter and inorganic nutrients from dairy wastewaters (Danalewich et al.,1998; Lateef et al., 2013; EPA,2008). Biological technologies for N and P removal are commonly accepted as the most economical and sustainable processes for wastewater nutrient bioremediation and they have been extensively applied for the treatment of industrial influents (Ferrera and Sánchez, 2016; Pholchan et al., 2010; EPA, 2007). However, traditional BNR processes are often multistage systems with significant infrastructural and financial demands. As a result, there is much ongoing research directed toward the identification, characterisation and application of novel microbial consortia facilitating more efficient BNR system design (Jetten et al., 1998; Dongen et al., 2001; Sliekers et al., 2002; Zeng et al., 2003a; Pynaert et al.,

2004; Kornaros et al., 2010; Pan et al., 2014; Gil-Pulido et al., 2018a). Intermittently Aerated Sequencing Batch Reactor (IASBR) technology has been developed as an enhancement to conventional Sequencing Batch Reactor (SBR) designs. IASBR is based on an activated sludge process but it differs from conventional BNR systems in that nitrogen and phosphorus removals are achieved in a single reactor, reducing both costs and footprint (Li et al., 2011; Pan et al., 2015). Various authors have reported high nutrient remediation capacity of the IASBR system during the treatment of various wastewaters demonstrating the potential application of the technology to dairy processing bioremediation (Li et al., 2008a, 2011; Zhan et al., 2011; Pan et al., 2014; Dong et al., 2016; Song et al., 2017). In recent studies, IASBR technology has been applied for the treatment of synthetic and industrial dairy processing wastewaters (Gil-Pulido et al., 2018a; Leonard *et al*., 2018a, 2018b) and removal efficiencies of over 90% have been reported for NH_4-N and PO_4-P, respectively.

Biological nutrient removal processes require at least three, distinct environmental conditions (i.e. anaerobic, anoxic and aerobic) to enrich for nitrifying, denitrifying, and anammox bacteria, respectively, in addition to polyphosphate-accumulating organisms (PAOs). These microbial groups collaboratively achieve the nutrient remediation function of the system (De Lucas *et al*., 2007). In this regard, the intermittent aeration strategy applied in IASBR technology (anaerobic/aerobic switch) provides appropriate environmental conditions for simultaneous N and P remediation in one single tank. A better knowledge of alternative pathways for nitrogen and phosphorus removal and the development of molecular methods have provided a more comprehensive understanding of bacterial community structure and dynamics in BNR processes (Ge et al., 2015b; van Loosdrecht et al., 2016; Ferrera et al., 2016; Guo et al.,2017). The application of molecular tools such as next generation sequencing (NGS) and investigations based on key functional genes involved in N and P metabolic pathways have allowed a greater understanding of key microbes remediating these inorganic nutrients. In recent years, new microbial groups such as denitrifying phosphorus accumulating organisms (DPAOs) have been identified in systems performing simultaneous nitrate/nitrite reduction and

phosphorus removal (Merzouki et al., 2001; Lee et al., 2001; Sun et al., 2015). DPAOs are capable of accumulating polyphosphate by using nitrate as an electron acceptor instead of oxygen and the organic carbon substrate can be used simultaneously for both phosphorus and nitrogen removal (Lee et al., 2001; Sun et al., 2015). The application of DPAOs offers the possibility of solving the oppose conditions needed with respect to sludge age for nitrogen and phosphorus removal (greater for nitrifiers than for PAOs) and competition for organic carbon source between denitrifiers and PAOs (van Loosdrecht et al., 1997; Ma et al., 2013; Sun et al., 2015). The discovery of DPAOs has provided an opportunity to design new types of wastewater treatment processes for the simultaneous removal of N and P with lower sludge production and more efficient use of COD (Kuba et al., 1996; Ahn et al., 2001; Zeng et al., 2003b; Tsuneda et al., 2006; Shi and Lee, 2006; Yang et al., 2010). These new findings highlight the importance of conducting molecular based investigations on bioreactor sludge microbial communities, particularly in novel reactor configurations, in order to continually advance their optimisation.

The performance of BNR systems rely on community structure and functionality, which can both be significantly affected by reactor operational factors and influent wastewater compositions (Wagner et al., 2002; Briones and Raskin, 2003; van Haandel and Van der Lubbe, 2007; Gentile et al., 2007; Lu et al., 2014; Rodríguez-Sánchez et al., 2019). For example, Gentile and co-workers previously assessed the effects of community structure on the stability of denitrification in chemostat systems (Gentile et al., 2007). They reported that predominant populations in the reactors differed depending on the nitrate and nitrous oxide levels in the influent. Several microbial ecology based studies performed on laboratory and full scale bioreactors to date, have tried to elucidate relevant links between system performance and community diversity under specific operational conditions (Liu et al., 2005; Gentile et al., 2007; Wells et al., 2009; Valentín-Vargas et al., 2012; Fan et al., 2017) and found that functional stability varied with community structure (Fernandez et al., 2000; Hashsham et al., 2000).

To date, characterization of the microbial ecology of an IASBR system during the treatment of dairy processing wastewaters has been limited to studies on

synthetic influent (Gil-Pulido et al., 2018a; Gil-Pulido et al., 2018b). However, it is crucial to evaluate such systems treating industrial dairy processing wastewaters to determine potentially important and relevant impacts on bacterial community structure, dynamics and performance. In this study, NGS and predictive metagenomic analyses of key functional genes were employed to characterize the bacterial community structure and functional profiles of two laboratory scale IASBRs during the treatment of synthetic and industrial dairy processing wastewaters. Additionally, a statistical approach by means of multivariate redundancy analysis (RDA) was used to identify operational and environmental factors that most significantly correlate with the dynamics of key bacterial groups.

3.2 Material and methods

3.2.1 Bioreactor set-up, operation and influent wastewater

Two laboratory scale IASBR systems were investigated in this study. They were operated at the Environmental Engineering laboratory in the Department of Civil Engineering, National University of Ireland, Galway (Fig. 1). The reactors were operated with a working volume of 8 litres each. They were located in a controlled-temperature room at 11 °C, in order to replicate average annual temperatures in Ireland. The two bioreactors were initially seeded with sludge from a full-scale conventional sequencing batch reactor (cSBR) at an Irish dairy processing factory.

The IASBR systems were operated in 12 hours cycle with an intermittent aeration strategy of four alternating periods of non-aeration (100 min) and aeration (60 min) per cycle. During the aeration periods, the aeration rate was set at 0.6 litres air min $^{-1}$. The choice of the applied aeration rate (0.6 L min $^{-1}$) followed optimal performance results obtained from previous investigations performed by the group (Gil-Pulido et al., 2018a).

At the beginning of each cycle, one litre of influent was pumped into each reactor and at the end one litre of treated wastewater was pumped out. The hydraulic retention time (HRT) was 4 days. After 80 days of bioreactor

stabilization, representative samples for metagenomic analyses were collected between days 81 and 167 of bioreactor operation. Initially, bioreactor 1 (IASBR 1) and bioreactor 2 (IASBR 2) treated synthetic wastewater modelled as described by Gil-Pulido et al. (2018a). The synthetic wastewater was prepared once every week and stored at 4°C. From day 89 onwards, IASBR 1 continued treating synthetic wastewater while IASBR 2 was switched onto industrial influent which was obtained from a partner dairy processing plant which had an on-site wastewater treatment plant. The dairy plant produced a variety of products such as casein, whey powder, butter and milk powder. The wastewater was taken from a balance tank preceded by a dissolved air flotation (DAF) system. Three different solid retention times (SRTs) were applied during the metagenomic based study period: 15,16 and 20 days. A summary of the operational conditions of both bioreactors is shown in Table 1.

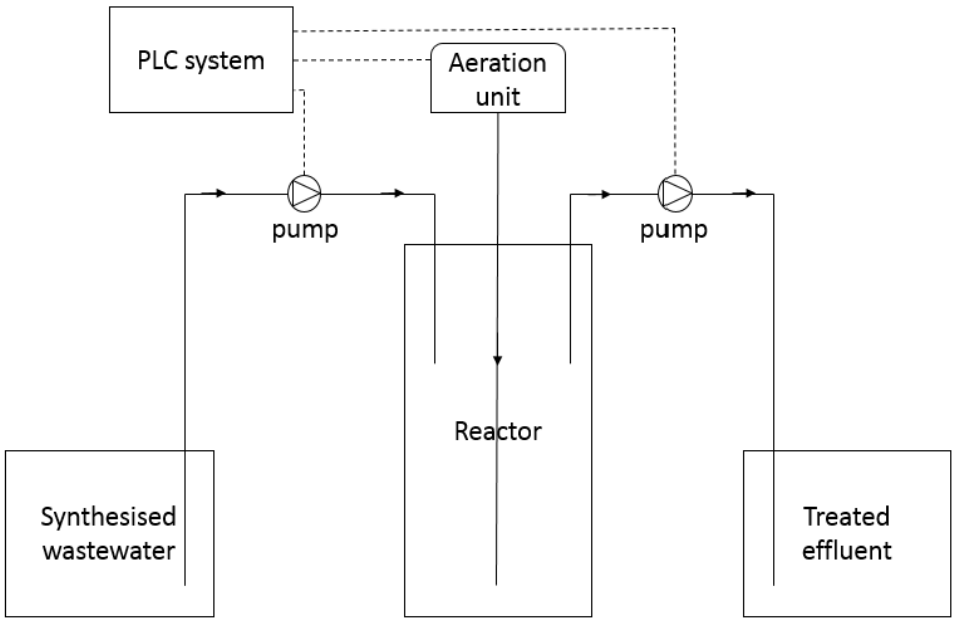

Figure 1. Schematic of the laboratory-scale IASBR system, (Gil-Pulido et al., 2018a).

Table 1. Operational parameters of the laboratory-scale IASBR systems from day 89 to 167 of operation. *SRT: Solids Retention Time; HRT: Hydraulic Retention Time; COD: Chemical Oxygen Demand; NH_4^+-N: ammonia nitrogen; PO_4^{3-}-P: ortho-phosphate.

Reactor	IASBR 1	IASBR 2
Wastewater type	Synthetic	Industrial
SRT (d)	15,16,20	15,16,20
HRT (d)	4	4
Temperature (°C)	11	11
Aeration rate (L min^{-1})	0.6	0.6
Total COD (mg L^{-1})	3483.8 ± 361.5	3835.7 ± 1044.7
Soluble COD (mg L^{-1})	3278.2 ± 351.0	2913.0 ± 593.7
NH_4^+-N influent (mg L^{-1})	32.8 ± 7.6	8.8 ± 9.6
PO_4^{3-}-P influent (mg L^{-1})	22.3 ± 2.3	11.8 ± 6.9
Cycle operation		
Aeration (min)	60	60
Non-aeration (min)	100	100

3.2.2 Wastewater analyses

Influent and effluent samples were analyzed regularly to test suspended solids (SS), dissolved oxygen (DO), chemical oxygen demand (COD) and biological oxygen demand (BOD_5) according to standardized analytical procedures (APHA, 2005). Quantification of NH_4^+-N, nitrite (NO_2-N), nitrate (NO_3-N), total oxidized nitrogen (TON), total nitrogen (TN), total phosphorus (TP) and PO_4^{3-}-P were analysed using a Konelab 20 Nutrient Analyser (Thermo Scientific), in accordance with the manufacturer's instructions.

3.2.3 Sample collection, DNA extraction and pyrosequencing

Activated sludge samples were routinely collected during the third aeration period within each IASBR cycle from day 81 until day 167. A subset of 5 different samples were selected from each bioreactor comprising representatives of each SRT. The sample on day 81 was considered as the reference sample after bioreactor stabilisation, prior to IASBR 2 receiving industrial influent. A summary of the samples used for metagenomic and community dynamics analyses is shown in Table 2. Samples were collected in sterile bottles and immediately placed at -20 °C until microbial diversity studies were performed at University College Cork, Ireland.

DNA extraction and 16S rRNA library preparation were performed as described by Gil-Pulido et al. (2018a). Universal primers U905F (5'-TGAAACTYAAAGGAATTG-3') and U1492R (5'-GGTTACCTTGTTACGACTT-3') with 10 nt unique barcodes were used to amplify the V5-V9 regions of bacterial and archaeal 16S rRNA genes from the extracted metagenomic DNA (Wang and Qian, 2009; Gao *et al*., 2015). The purified products were pooled in equimolar quantities and forwarded to MACROGEN (Seoul) for emulsion PCR and 454 GS FLX+ pyrosequencing service.

Table 2. Summary of analysed samples for metagenomic and ecological analyses. * Represents initial biomass prior industrial switch on day 89 of bioreactor operation.

Reactor	Sample code	Day of operation since starting	Influent type	SRT (d)
IASBR 1	R1 (d 81)	81	Synthetic	15
IASBR 1	R1 (d 94)	94	"	20
IASBR 1	R1 (d 112)	112	"	20
IASBR 1	R1 (d 140)	140	"	16
IASBR 1	R1 (d 167)	167	"	16
IASBR 2	R2 (d 81)	81*	Synthetic	15
IASBR 2	R2 (d 94)	94	Industrial	20
IASBR 2	R2 (d 112)	112	"	20
IASBR 2	R2 (d 140)	140	"	16
IASBR 2	R2 (d 167)	167	"	16

3.2.4 Sequence data analysis and taxonomy assignment

The raw 16S rRNA gene fragments (reads) were corrected using Acacia (Bragg *et al*., 2012). Subsequent analyses were carried out using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). Chimeras were filtered out and removed using USEARCH v6.1 (Edgar 2010). Quality-filtered sequences (minimum length 200 bp, with no ambiguous bases and homopolymers of 6 bp as maximum) were aligned using PynAST against the SILVA 128 reference database (Quast *et al*., 2012). Taxonomy was assigned to each OTU using the RDP classifier at a 0.97 threshold. The filtered alignments were clustered into OTUs at the 97% identity level in an open-reference OTU picking process implemented in QIIME.

Raw sequences were submitted to GenBank, Sequence Read Archive (SRA), under accession number PRJNA509602.

3.2.5 Taxonomy graphs and heat maps

For each sample, a bar graph was generated defining the differences in community structure at phylum and genus level. The bar graphs were based on the relative abundance ≥ 1.5 % in at least one sample and created by GraphPad Prism program v.5. Morisita index and Similarity Percentage (SIMPER) analyses results were represented by heat maps created with Microsoft Excel.

3.2.6 Biostatistical analyses

Ordination of bacterial community composition in the initial biomass and the bioreactors was performed via principal components analysis (PCA) using R and RStudio (RStudio R.T. 2015; Team R.C. 2018). Subsequently, species richness indices (ACE and Chao 1) and true diversity (unique OTUs) of bioreactor samples were calculated according to QIIME pipeline procedures with a normalized OTU table using the cumulative sum scaling (CSS) technique (Paulson et al., 2013). Similarity between bioreactor samples was determined by comparing OTU presence and abundance using the Morisita index for similarity (Van der Wielen *et al*., 2009; Wolda et al., 1981) measured by PAlaeontological STatistics (PAST) version 3 software (Hammer et al. 2001). Morisita index range from 0 to 1, with 1 indicating complete (100%) similarity of two communities.

Analysis of differential abundance of key microbial communities between the two different bioreactors at genus taxonomy level were computed using DESeq2 program with $padj < 0.05$ (Love et al., 2014).

The influence of environmental parameters over the ecological composition of the IASBRs was calculated using redundancy analyses (RDA). First, the average percent contribution of taxonomy at genus level to the dissimilarity between the bioreactors was calculated using Similarity Percentage (SIMPER) analysis (Clarke, 1993) in PAST 3. The results were then filtered at ≥ 1% cut-off prior to performing the RDA analyses. Then, the statistical significance of wastewater type and SRT was assessed by one-way

PERMANOVA (Bray-Curtis distance) analysis in PAST 3. An environmental variable was considered statically robust at a *p-value* ≤ 0.05. For RDA analyses, the data from significant environmental parameters (wastewater type, NH_4-N and PO_4-P) were normalized to the LOG (X+1) transformation; then, the OTU distribution and the normalized environmental parameters data were used to calculate a multivariate redundancy analysis by 499 unconstrained Monte-Carlo simulations under a full permutation model through CANOCO version 4.5 (Ter Braak et al., 2002).

3.2.8 Prediction of functional metagenome in the biological samples

The functional metabolic profiling of bacterial communities in the activated sludge bioreactor samples was predicted using the PICRUSt program (Langille et al., 2013). Firstly, Greengenes 13_5 reference dataset was used for close-reference OTU picking in QIIME and the resulting OTU table was normalized using DESeq2 in R (Love et al.,2014*;* Team R.C., 2018). The DESeq2 normalized table was then normalized by predicted 16S copy number abundance. Secondly, the functional metagenome prediction was performed using PICRUSt, based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs (KOs). The Nearest Sequenced Taxon Index (NSTI) cut-off of < 1.5 was applied for the quality control assessment of the functional predictions. Finally, the top contributors for the nitrogen and phosphorus related genes (Nitrogen: *hao, nrfA, nirK, norB, nosZ, narG, narZ, nxrA;* Phosphorus*: ppK, ppx, phaC*) were computed in PICRUSt and further processed for family level contribution using R, with plyr package for data manipulation (Wickham H., 2011; Langille et al., 2013; Team R.C., 2018).

3.3 Results and discussion

3.3.1 Overall performance of the bioreactors

Post stabilisation, the two bioreactors were operated for 87 days under the operational conditions shown in Table 1 in an intermittent aeration strategy. Total COD (COD_t) concentrations in the industrial influent had more variations than in the synthetic while soluble COD (COD_{Sol}) values were similar. The soluble COD (COD_{Sol}) removal was stable and efficient (≥ 95%) during this period for both bioreactors. The COD_{Sol} effluent concentrations were 105.7 ± 27.0 mg L^{-1} and 145.4 ± 23.5 mg L^{-1} for IASBR 1 and IASBR 2 respectively, indicating that the remainder was likely to be as a result of colloidal particles such as soluble microbial products. The total COD (COD_t) removal showed lower but comparable achieved efficiencies in both bioreactors: 75.0 % ± 2.0 for IASBR 1 and 71% ± 11.2 for IASBR 2.

It was noted that the synthetic influent had 32.8 ± 7.6 mg L^{-1} NH_4^+-N on average, while the NH_4^+-N in the industrial influent was considerably lower at 8.8 ± 9.6 mg L^{-1}. Similarly, industrial influent had 11.8 ± 6.9 mg L^{-1} PO_4^{3-} -P on average while orthophosphate in the synthetic influent was higher (22.3 ± 2.3 mg L^{-1} PO_4^{3-} -P). NH_4^+-N removal was observed to be higher and more stable in IASBR 1 (94.7± 9.2) in comparison with the removal efficiency in IASBR 2 (66.2 ± 40.3) (Table 1). Nutrient removal performance of ammonia during the synthetic treatment was observed to be comparable to results observed in IASBR laboratory scale bioreactors treating dairy processing and slaughterhouse wastewaters at low temperature and at an aeration rate of 0.6 L min^{-1} (Leonard et al., 2018b; Pan et al., 2014). PO_4^{3-} -P removal efficiencies were 63.5 ± 9.7 and 71.2 ± 14.7 for IASBR 1 and IASBR 2, respectively and were observed to be lower than those reported in similar studies (Gil-Pulido et al., 2018a). Differences in nutrient removal efficiencies for both bioreactors suggested that the system might be sensitive to shock loadings/nutrient variation observed in the industrial influent. Alternatively, varying C: N: P ratios in IASBR 2 may drive detrimental changes in the microbial community, thereby impacting on performance.

3.3.2 Cluster analysis of initial biomass and IASBRs samples

Principal components analysis (PCA) was conducted to evaluate similarities between the initial biomass and the IASBR samples. The PCA results at OTU level are shown in Figure 2. PCA plots showed that bioreactor samples formed two strongly different clusters from the initial biomass. This suggests that existing bacterial populations in the seed sludge evolved differently according to the new bioreactor conditions. Moreover, it is hypothesized that the relative population of microorganisms of the bioreactors may be influenced by the composition of the influent wastewater (De Lucas et al., 2007, Lee et al., 2015; Langenheder et al., 2011).

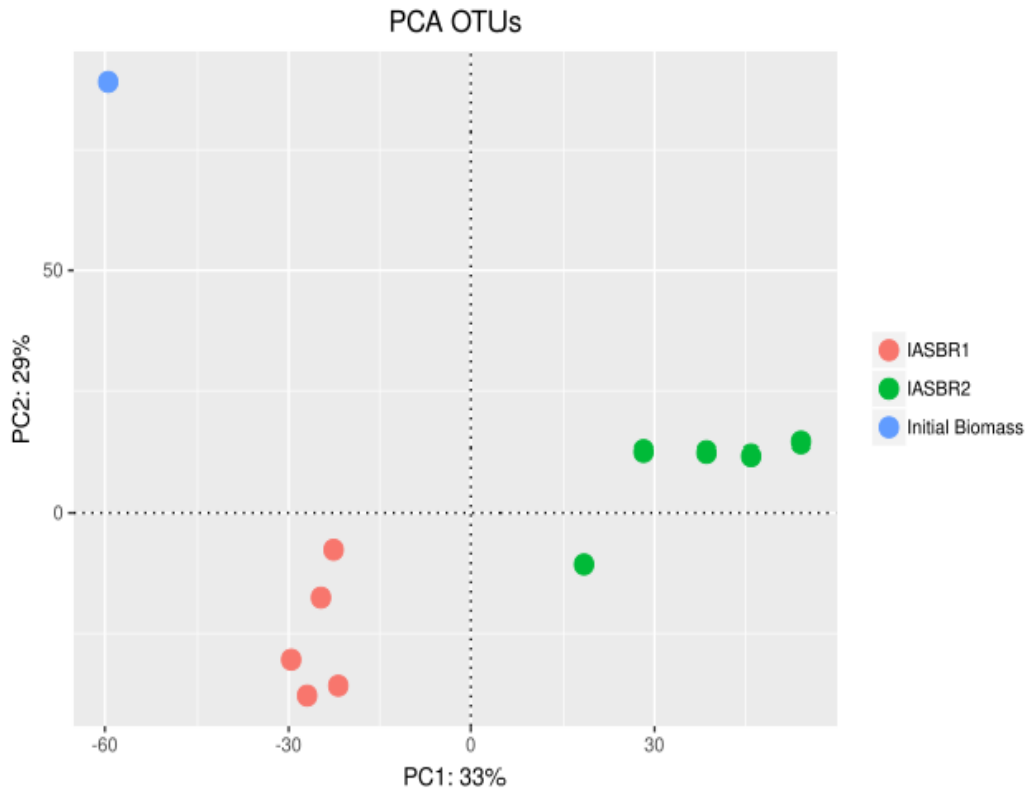

Figure 2. Principal components analysis (PCA) at OTU level of the initial biomass and bioreactors (IASBR 1 and IASBR 2) samples

3.3.3 α-Diversity indices and similarity of bioreactors samples

The bacterial diversity based on ACE, Chao 1 and unique OTUs are summarized in Table S1. On the basis of true diversity (Unique OTUs) and species richness (ACE and Chao 1) the activated sludge samples from IASBR 2 revealed the greatest diversity when compared to IASBR 1. For IASBR 1, the patterns of ACE, Chao1 and Unique OTUs were identical and displayed considerably less diversity. This suggests that the microbial consortium enriched in the IASBR systems by synthetic substrates are distinct from those enriched by industrial wastewater (Coats et al., 2017).

The Morisita similarity index between the dominant communities in the two bioreactors (Fig. S2) was 0.67 after bioreactor stabilization (day 81), indicating that bacterial community development was not identical during the first 80 days of operation. From day 94, the Morisita index between IASBR 1 and IASBR 2 slightly decreased to 0.56, suggesting dissimilar bacterial community structure upon switching to industrial influent.

3.3.4 Microbial ecology structure of the initial biomass and the IASBRs samples

Taxonomic classification at phylum and genus level for the initial biomass and bioreactor samples are shown in Figures 3 and 4. Graphs indicated that the bacterial community composition in the initial biomass and characterized bioreactor samples (Table 2) were dominated by different groups of bacteria. The most predominant genera in the initial biomass belonged to the *Chitinophagaceae* family within the *Bacteroidetes* phylum. Uncultured *Chitinophagaceae* accounted for 35.7% relative abundance in the initial biomass but was negligible thereafter (<0.2% relative abundance) in both bioreactors. The same trend was observed with other genera identified in the initial biomass such as for example *Terrimonas*, *Veillonella*, *Candidatus Competibacter*, *NS9 Marine group* representatives or *Streptococcus*, which all displayed <0.1% abundance within the bacterial community profiles of

IASBR 1 and IASBR 2. Taxonomy composition results of the initial biomass and the bioreactors support cluster analyses performed by PCA indicating the adaption of the seed biomass to the IASBR configuration (intermittently aerated cycles) and the operational conditions applied in this study.

Over 70% of the total microbial community in IASBR 1 was composed of *Proteobacteria* phylum followed by *Bacteroidetes* (19% abundance) while in IASBR 2 *Proteobacteria* and *Bacteroidete*s phyla accounted for 35% and 41% abundances, respectively. Some recent studies (Regueiro et al., 2014; Gonzalez-Martinez et al., 2018) have shown that the *Proteobacteria* and *Bacteroidetes* phyla may be important in the case of a low-temperature shock (Keating et al., 2016). For both bioreactors, *Betaproteobacteria* was the dominant class within the *Proteobacteria* phylum with 67% and 23% relative abundances in IASBR 1 and IASBR 2, respectively. The observed results are similar to previous reported findings, where *Proteobacteria* and *Betaproteobacteria* were the most predominant phyla and class, respectively, in activated sludge communities and in simultaneous nitrogen and phosphorus removal systems (Juretschko et al., 2002; Yu and Zhang, 2012; Guo et al., 2017).

Genus-level identification revealed the dominance of *Comamonadaceae*-related members during the treatment of synthetic influent (IASBR 1) and more diverse microbial population profiles when industrial wastewater was introduced (IASBR 2) (Fig. 4). *Polaromonas* and other *Comamonadaceae* groups were the most predominant genera in IASBR 1, accounting for 11% and 53% of relative abundance on average, respectively. The results are similar to previous investigations reported by Gil-Pulido *et al*. (2018a) where *Comamonadaceae* family members dominated the ecological profile of an IASBR system treating synthetic dairy wastewater under conditions of low aeration and low temperature (Gil-Pulido et al., 2018a, 2018b). Interestingly, during the treatment of industrial influent (IASBR 2) the presence of other *Comamonadaceae* decreased to 9.1% on average and *Polaromonas* was detected in less than 0.5% relative abundance. *Polaromonas* and *Comamonadaceae* related-member dominance during synthetic influent treatment suggests that the presence of those groups may vary significantly depending on influent characteristics. The observed *Polaromonas* profile in

IASBR 1 was comparable with the trend followed by *Hydrogenophaga* in IASBR 2. While *Polaromonas* abundance increased in IASBR 1 from day 94 up to 16% and decreased in IASBR 2, *Hydrogenophaga* increased in IASBR 2 up to 19% relative abundance following the switch to industrial influent. Analysis of differential abundance results showed that differences in *Polaromonas*, *Hydrogenophaga* and other *Comamonadaceae* abundances between both bioreactors were statistically significant (Table S2). The family *Comamonadaceae* constitute one of the major populations of denitrifying clusters (Wu et al., 2013) and some of its members have been reported to be capable of performing simultaneous nitrification/denitrification and phosphorus removal (Khan et al., 2002; Khardenavis et al., 2007; Ge et al., 2015a; Gil-Pulido et al., 2018a, 2018b). In addition, the genus *Polaromonas* has been reported to have denitrifying metabolism (Beganskas *et al*., 2018) and to promote biological nutrient removal, comparable to *Flavobacterium* or *Thauera* (Gil-Pulido et al., 2018b). Our results suggest that *Polaromonas*, *Hydrogenophaga* and other *Comamonadaceae*-related members may potentially play and important role in IASBR systems during simultaneous nitrogen and phosphorus removal. Other functional microorganisms from BNR related systems were also observed. For example, *Thauera* was identified in both systems, being more abundant in IASBR 2 than in IASBR 1 (Fig. 4). *Thauera* have been described as one of the dominant species of denitrifiers in nitrogen removal systems (Lu *et al*., 2014; Fan *et al*., 2017) and as a potential denitrifying phosphorus-accumulating bacteria (Sun et al., 2015). *Leadbetterella*, which was also identified in IASBR2 has been previously reported among the main bacterial genera in partial nitrification systems with potential functional significance (Wang *et al*., 2016). Uncultured species of both *Thauera* and *Leadbeterrella* genera have also been reported in biological nitrogen removal bioreactors operating at low temperatures (Yao et al., 2013). Interestingly, our observations suggest that the IASBR system contained representatives of well-established nutrient removal bacterial in addition to less well characterised, but potentially important organisms for BNR processes.

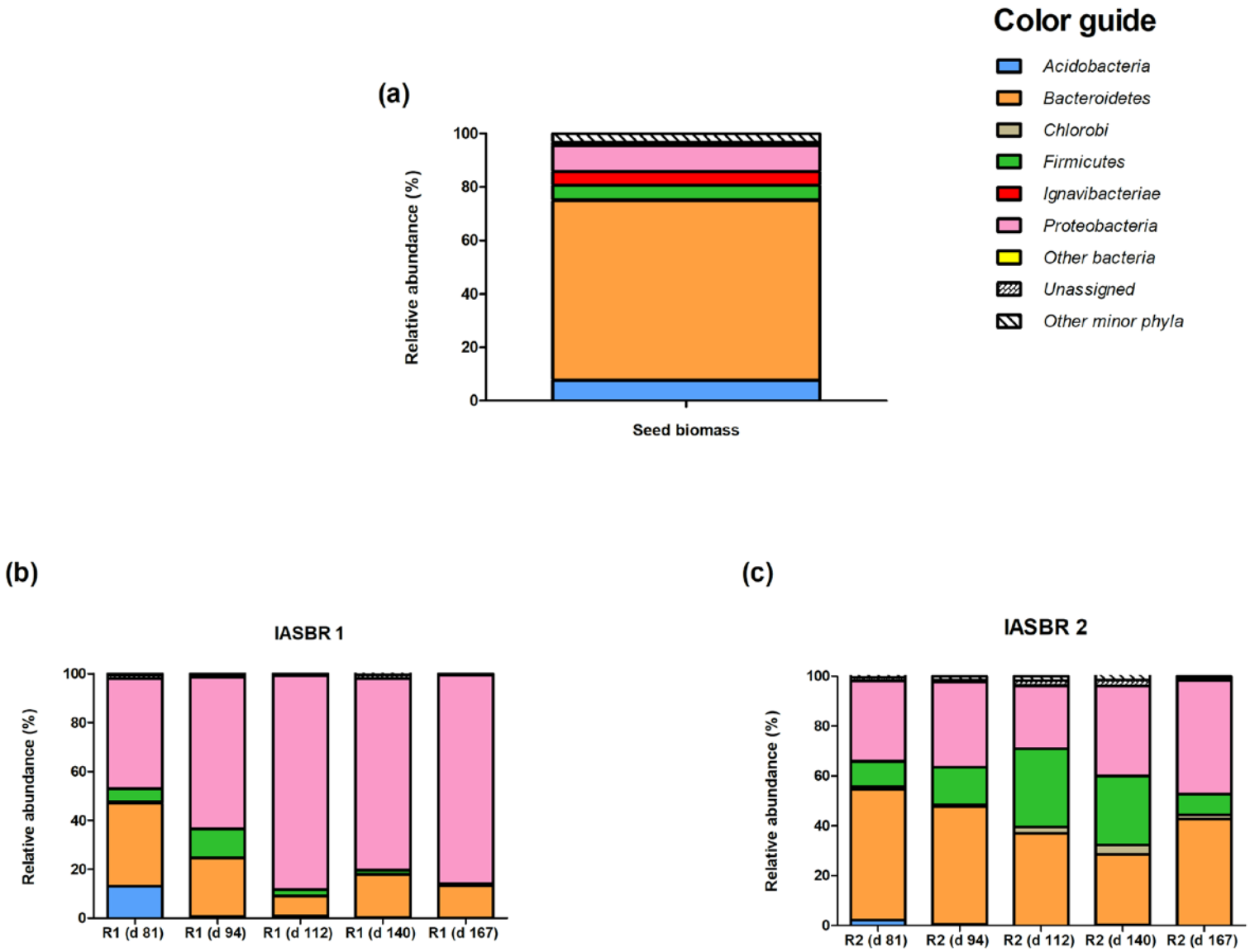

Figure 3. Bacterial community composition at phylum level of: (a) seed biomass, (b) IASBR 1 and (c) IASBR 2. Sample on day 81 represents the biomass prior industrial switch in IASBR 2 on day 89 of bioreactor operation.

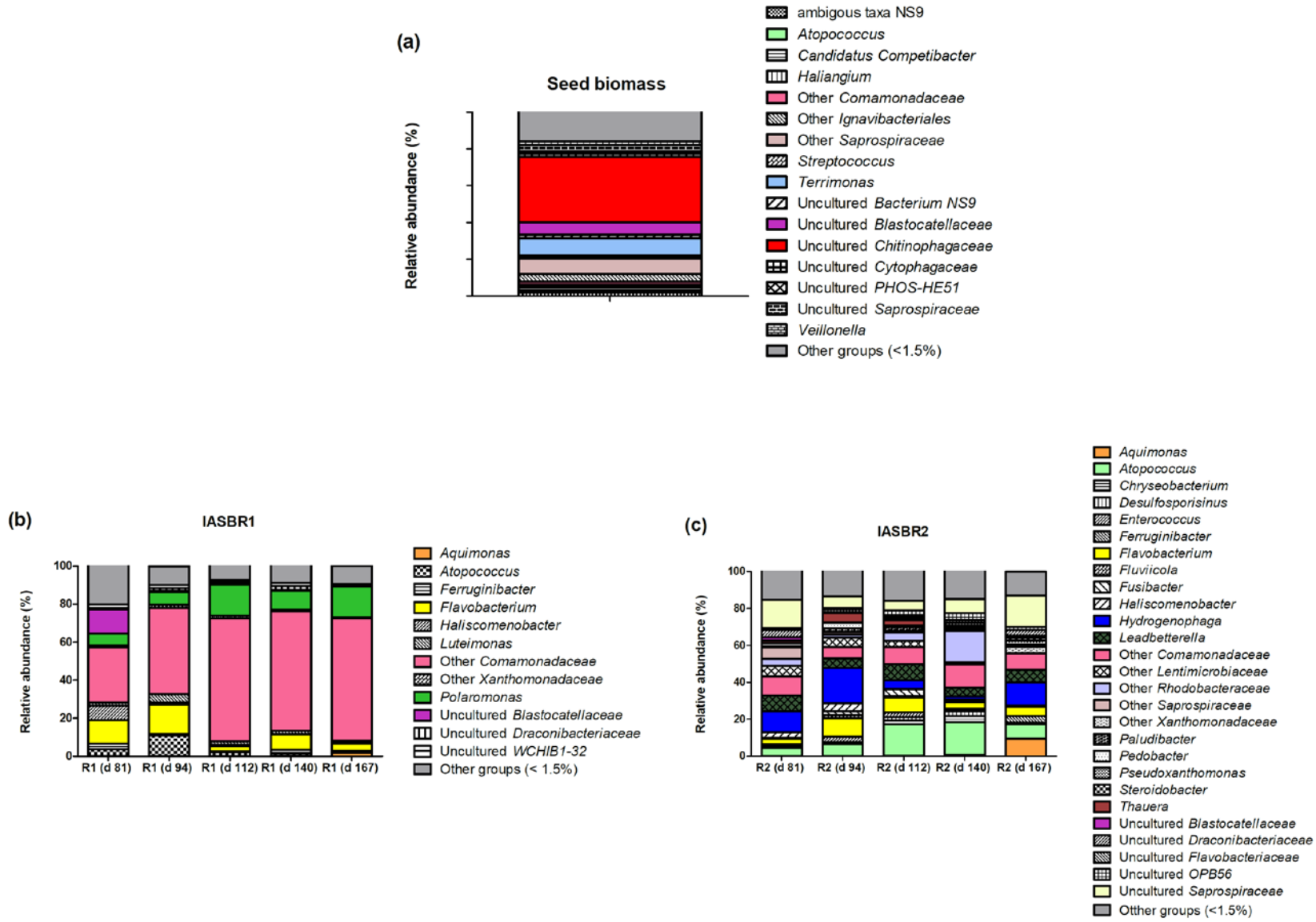

Figure 4. Bacterial community composition at genus level of: (a) seed biomass, (b) IASBR 1 and (c) IASBR 2. Sample on day 81 represents the biomass prior industrial switch in IASBR 2 on day 89 of bioreactor operation.

The taxonomical analyses performed on the initial biomass and bioreactor samples revealed the lack of nitrifiers and major PAOs reportedly associated with biological nutrient removal systems (Crocetti et al., 2000; Ge et al., 2015b). The primary functional nitrifying bacteria in wastewater treatment plants, AOB and NOB, were not found in the analyzed samples. *Nitrospira*, within *Nitrospiraceae* family, was detected in the initial biomass with 0.1% relative abundance but their presence was not sustained within subsequent bioreactor samples. This correlates with previous studies where nitrifying bacteria were reported to be present in low abundance in co-mixing systems (Li et al., 2011). These taxonomic findings were supported with predictive metagenomic analyses performed on bioreactor samples where ammonia monooxygenase genes (*amoA*), linked to traditional ammonia oxidation were not detected (Table 3). The most important PAOs described to date include *Candidatus* Accumulibacter phosphatis (Crocetti et al., 2000; He and McMahon, 2011) and genus *Tetrasphaera* (Kristiansen et al., 2013; Marques et al., 2017). *Candidatus* Accumulibacter phosphatis, a *Rhodocyclaceae*-related member, was detected in the initial biomass with 0.2% abundance and in less than 0.1% relative abundance on average in IASBR 1. It was not detected during industrial influent treatment (IASBR 2). These findings suggested that IASBR systems do not favour the proliferation of well described nitrifiers and major PAOs which may be related to the bioreactor operational conditions. Glycogen-accumulating organisms (GAOs) are a group of microorganisms detected in EBPR systems which do not contribute to phosphorus removal but outcompete PAOs (Erdal et al., 2003). In this study, the presence of known GAOs was observed in the initial biomass and in IASBR 2 (Fig. 3 and Fig. 4). *Defluviicoccus* (Oehmen et al., 2006) was found only in the initial biomass at 0.2% abundance and was not detected in bioreactor samples. *Candidatus Competibacter* (McIlroy et al., 2014) was observed in the initial biomass with 1.7% abundance but was not detected in biomass samples from the latter stages of IASBR 2 operation. The reported absence or low abundance of traditional key biological nutrient removal performers such as *Nitrospira* or *Candidatus* Accumulibacter phosphatis in the characterized IASBR biomass samples highlights the importance of investigating microbial ecology underpinning IASBR systems to potentially

highlight novel microorganisms involved in nitrogen and phosphorus removal pathway.

Table 3. Predictive analysis of genes involved in nitrogen and phosphorus removal metabolisms at family taxonomy level and relative contributions to genes of interest. Tables 3.1 (a) and (b) represents IASBR 1. Tables 3.2 (a) and (b) represents IASBR 2.

Table 3.1 (a). Nitrogen genes in IASBR 1

Genes	Taxonomy	Relative contribution (%)
amoA	*No detected*	-
hao	*c__OPB56_o___f__*	61.98
	o__CL500-15_f__	15.02
	o__Ignavibacteriales_f__Ignavibacteriaceae	9.41
	o__Bdellovibrionales_f__Bacteriovoracaceae	7.57
nrfa	*c__OPB56_o___f__*	51.24
	o__Cytophagales_f__Cyclobacteriaceae	14.61
	o__Ignavibacteriales_f__Ignavibacteriaceae	7.78
	o__Bdellovibrionales_f__Bacteriovoracaceae	6.26
nirK	*o__Xanthomonadales_f__Xanthomonadaceae*	61.17
	o__Rhodocyclales_f__Rhodocyclaceae	24.11
	o__Flavobacteriales_f__[Weeksellaceae]	4.95
	o__Flavobacteriales_f__Flavobacteriaceae	3.02
norB	*o__Burkholderiales_f__Comamonadaceae*	81.63
	o__SC-I-84_f__	12.06
	o__Xanthomonadales_f__Xanthomonadaceae	3.44
	o__Rhodocyclales_f__Rhodocyclaceae	1.79
nosZ	*o__Burkholderiales_f__Comamonadaceae*	85.61
	o__[Saprospirales]_f__Saprospiraceae	6.17
	o__Rhodocyclales_f__Rhodocyclaceae	3.34
	o__Flavobacteriales_f__Flavobacteriaceae	1.42
narG, narZ, nxrA	*o__Burkholderiales_f__Comamonadaceae*	85.03
	o__SC-I-84_f__	12.55
	o__Rhodocyclales_f__Rhodocyclaceae	1.86
	o__SC-I-84_f__	11.85
	o__Xanthomonadales_f__Xanthomonadaceae	2.49
	o__Rhodocyclales_f__Rhodocyclaceae	1.73

Table 3.1 (b). Phosphorus genes in IASBR 1		
Genes	**Taxonomy**	**Relative contribution (%)**
ppk	*o__Burkholderiales_f__Comamonadaceae*	72.15
	o__SC-I-84_f__	10.31
	o__Xanthomonadales_f__Xanthomonadaceae	4.33
	o__[Saprospirales]_f__Saprospiraceae	3.10
ppx	*o__Burkholderiales_f__Comamonadaceae*	73.11
	o__SC-I-84_f__	10.59
	o__Xanthomonadales_f__Xanthomonadaceae	4.44
	o__Flavobacteriales_f__Flavobacteriaceae	2.47
phaC	*o__Burkholderiales_f__Comamonadaceae*	83.08
	o__SC-I-84_f__	11.85
	o__Xanthomonadales_f__Xanthomonadaceae	2.49
	o__Rhodocyclales_f__Rhodocyclaceae	1.73

Table 3.2 (a). Nitrogen genes in IASBR 2		
Genes	**Taxonomy**	**Relative contribution (%)**
amoA	No detected	-
hao	*c__OPB56_o___f__*	84.15
	c__TSBW08_o___f__	9.62
	o__Desulfovibrionales_f__Desulfomicrobiaceae	3.79
	o__Ignavibacteriales_f__Ignavibacteriaceae	1.41
nrfa	*c__OPB56_o___f__*	73.05
	c__TSBW08_o___f__	8.35
	o__Flavobacteriales_f__[Weeksellaceae]	7.23
	*k__Bacteria_p__Proteobacteria_*	3.29
nirK	*k__Bacteria_p__Proteobacteria_*	35.61
	o__Rhodocyclales_f__Rhodocyclaceae	21.33
	o__Xanthomonadales_f__Xanthomonadaceae	16.65
	o__Flavobacteriales_f__Flavobacteriaceae	12.11
norB	*o__Burkholderiales_f__Comamonadaceae*	58.69
	o__Rhodocyclales_f__Rhodocyclaceae	13.79
	o__Rhodobacterales_f__Rhodobacteraceae	11.36
	o__Xanthomonadales_f__Xanthomonadaceae	5.94
nosZ	*o__[Saprospirales]_f__Saprospiraceae*	26.35
	o__Rhodocyclales_f__Rhodocyclaceae	22.75
	o__Rhodobacterales_f__Rhodobacteraceae	21.81
	c__OPB56_o___f__	20.13
narG, narZ, nxrA	*o__Burkholderiales_f__Comamonadaceae*	77.79
	o__Rhodocyclales_f__Rhodocyclaceae	18.19
	o__Rhodobacterales_f__Rhodobacteraceae	6.15
	o__Xanthomonadales_f__Xanthomonadaceae	4.66

Table 3.2 (b). Phosphorus genes in IASBR 2		
Genes	**Taxonomy**	**Relative Contribution (%)**
ppk	*o__Burkholderiales_f__Comamonadaceae*	30.31
	o__[Saprospirales]_f__Saprospiraceae	10.95
	o__Flavobacteriales_f__Flavobacteriaceae	8.04
	o__Cytophagales_f__Cytophagaceae	7.50
ppx	*o__Burkholderiales_f__Comamonadaceae*	27.24
	o__Cytophagales_f__Cytophagaceae	13.45
	o__Rhodobacterales_f__Rhodobacteraceae	10.32
	o__Flavobacteriales_f__Flavobacteriaceae	7.24
phaC	*o__Burkholderiales_f__Comamonadaceae*	65.20
	o__Rhodocyclales_f__Rhodocyclaceae	18.77
	o__Rhodobacterales_f__Rhodobacteraceae	6.15
	o__Xanthomonadales_f__Xanthomonadaceae	4.66

3.3.5 Linkage of bacterial community structure and operational parameters

The PERMANOVA analyses performed on bacterial community structure and operational parameters showed that wastewater type, NH_4^+-N and PO_4 -P contents had a statistically significant influence ($p < 0.05$) over the bacterial community structure present in the bioreactors (Table 4). Other studied environmental factors such as COD_t and SRT that may explain variations in the microbial community structure did not show statistical significance in the current study ($p > 0.05$). Surprisingly, SRT was not selected as an important variable ($p > 0.05$) influencing the variability of the communities in the two systems. This result may suggest the SRT variations were not imposed for sufficiently long periods of time to result in significant changes in the communities during the period of study (see the range of SRT in Table 2). SRT has previously been shown to have a strong impact on community structure and diversity (Ahmed et al., 2007; Huang et al., 2001; Rodriguez-Sanchez et al., 2014; Hai et al., 2014). It is widely reported that low retention

times are more effective for phosphorus removal and longer SRTs are suitable for slow growing autotrophic bacteria (AOB and NOB) responsible for nitrogen removal (Silva et al, 2012; Ge et al., 2015a; Ge et al., 2015b). A satisfactory balance must be achieved to meet the requirements of these functional groups for optimal removal of both nutrients by means of IASBR technology.

Table 4: Permutational multivariate analysis of variance (PERMANOVA) results for the investigated environmental parameters. a) significance of the main operational variables of the study over the ecological composition of the bioreactors; b) significance of the key operational variables depending on wastewater type. *means significantly different ($p<0.05$).

a)

Variable	F-statistic	*p-value*
NH_4-N	41.23	0.0001*
PO_4-P	23.90	0.0002*
COD_t	0.93	0.3587

b)

Variable	F-statistics	*p-value*
Wastewater type	5.3990	0.0089*
SRT	0.9109	0.5366

The correlation between dominant genera based on SIMPER analysis results (Fig. S2) and statistically significant operational conditions of the bioreactors (Table 4) were assessed through calculation of multivariate redundancy analysis (RDA) (Fig. 6). SIMPER analysis identified the top 15 genera (≥ 1%

dissimilarity contribution) that primarily contributed to the difference between the bacterial profiles in IASBR 1 and IASBR 2. According to SIMPER heat map results, *Comamonadaceae* related members were identified as being the main contributors to the total dissimilarity between bioreactors.

Figure 5 shows the different bacterial clusters with respect to the environmental conditions analyzed. *Polaromonas*, other *Comamonadaceae*, *Flavobacterium* and *Luteimonas* were positively correlated with influent NH_4^+-N and PO_4^--P concentrations and synthetic wastewater while negatively associated with industrial influent. These correlations corresponded with their relative abundances observed in IASBR 1 and IASBR 2 (Fig. 3 and Fig. 4). The affinity of *Polaromonas*, *other Comamonadaceae* and *Luteimonas* for higher ammonium in the influent might explain their decreased abundance in IASBR 2 compared to their presence in IASBR 1. *Hydrogenophaga* and *Saprospiraceae* (uncultured*)* showed a negative relationship with the concentration of PO_4^--P in the influent and a positive correlation with the industrial substrate. The increase in *Hydrogenophaga* and *uncultured Saprospiraceae* abundances during the treatment of industrial influent (Fig.5) maybe related to the results observed for those genera in the RDA plots. The same trend was observed for *Leadbetterella*, *Atopococcus, other Lentimicrobiaceae* and *Thauera* genera that were favored by industrial wastewater type. Several studies have demonstrated that variations in key microbial communities in biological nutrient removal processes may react to varying influent characteristics (Hu et al., 2003; Wells et al., 2009; Lu et al., 2014; Ge et al., 2015b; Gonzalez-Martinez et al., 2016; Che et al., 2017). In the current study, RDA results suggested that the occurrence of specific bacterial species observed in the ecological profiles of IASBR 1 and IASBR 2 were influenced by variations in NH_4^+-N and PO_4^{3-} -P concentrations.

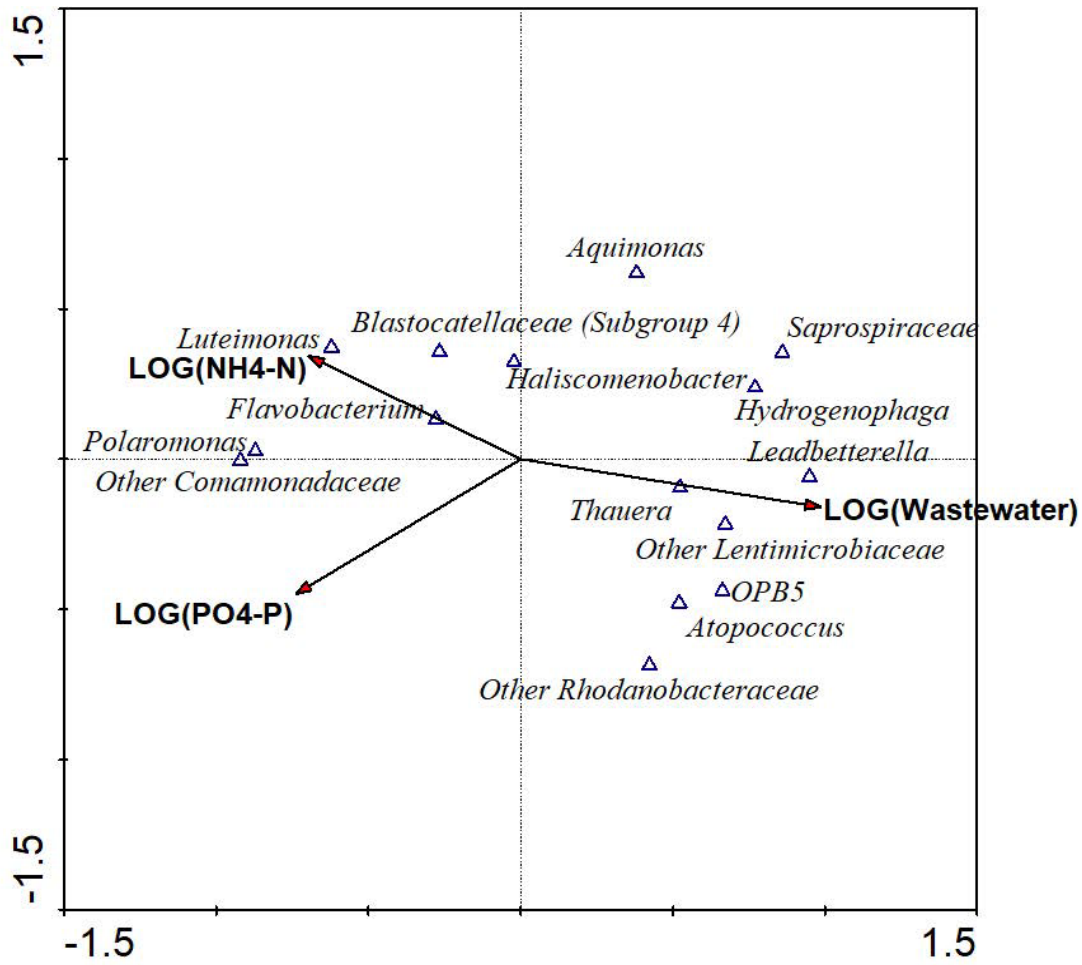

Figure 5. Redundancy analysis (RDA) of influent characteristics and genera contributing to the dissimilarity (based on SIMPER analysis, Table S2) of the bioreactors (IASBR 1 and IASBR 2). Only the statistically significant ($p < 0.05$) explanatory variables (operational parameters) are represented. Operational parameters are represented by arrows, and bacterial genera are represented by triangles. Arrows indicate the direction and strength of variables correlated with bacterial community structure

3.3.6 Functional gene prediction involved in nitrogen and phosphorus removal

OTUs contributing to key enzymes involved in nitrogen and phosphorus removal in wastewater treatment systems, as defined by Lu et al., 2014, Fan et al., 2017 and Gil-Pulido et al., 2018a, were further investigated by PICRUSt. The top 4 family contributors (≥1%) to the genes of interest in each

bioreactor are listed in Table 3. PICRUSt analysis indicated that the *Comamonadaceae* family was the top contributor to phosphorus removal genes of interest in both bioreactors. In IASBR 1, *Comamonadaceae* potentially contribute > 70% of the *ppk* (polyphosphate kinase) and *ppx* (exopolyphosphatase) and > 80% of the *phaC* (PHA synthase) genes. The contribution of the family to *ppk*, *ppx* and *phaC* in IASBR 2 was observed to be lower than 35% for *ppk* and *ppx* and ~ 65% for *phaC*. The potential of *Comamonadaceae* family in biological phosphorus removal processes has been recently investigated (Ge et al., 2015a; Gil-Pulido et al., 2018a). Ge and co-workers (2015a) found *Comamonadaceae*-related members consistently represented in the microbial ecology profile of an SBR treating abattoir wastewater. Further analyses combining fluorescence *in situ* hybridization (FISH) and DAPI staining revealed that *Comamonadaceae* was a strong candidate responsible for the high-rate Bio-P removal process (Ge et al., 2015a). Recent work in our group, has demonstrated the functional capacity for Bio-P accumulation in *Polaromonas naphthalenviorans CJ2* type strain exposed to pH 5.5 which has previously been shown to induce *ppK* expression in a wide array of bacteria (McGrath and Quinn,2000) (results not shown). These findings along with the observed PICRUSt results in the current study, suggest the need for future investigations focused on studying the role of *Comamonadaceae*-related members in phosphorus removal processes. Other families with the potential to contribute to phosphorus removal such as *Xanthomonadaceae*, *Saprospiraceae, Rhodocyclaceae, Flavobacteriaceae*, *Cytophagaceae* and *Rhodobcteraceae* were also observed in the profile of contributors to the phosphorus genes of interest in IASBR 1 and IASBR 2. Members of these dominant families have been previously reported to be involved in phosphorus remediation processes in different bioreactor configurations (Kong et al., 2007; Kamika et al., 2014; Valverde-Perez et al., 2016; Xin et al., 2016; Gao et al., 2017). The diversity of bacterial groups contributing to key functional genes in the phosphorus pathway was observed to be higher in IASBR 2 than in IASBR, which suggests the potential impact of the industrial influent on bacterial diversity.

With respect to nitrogen genes, PICRUSt analysis revealed that nitrification genes found in the samples were *hao* (hydroxylamine oxidoreductase) and *nrfa* (nitrite reductase). Ammonia oxidation genes (*amo*A) were not found in any of the samples. These results may suggest the potential of heterotrophic nitrification metabolism occurring in the bioreactors (Rodriguez-Sanchez et al*.,* 2018). PICRUSt results revealed the high abundance of genes involved in nitrate reduction processes, e.g. *nirK* (copper-containing nitrite reductase), *norB* (nitric oxide reductase subunit B) and *nosZ* (nitrous oxide reductase*).* Genes encoding denitrification reductases have been used as functional biomarkers in the detection of bacterial populations with denitrification capabilities (Lu et al., 2014). Many dominant phylotypes could potentially contribute to denitrification processes occurring in IASBR systems. *Comamonadaceae* family contributed most significantly to *nosZ*, *norB* and *narG* (nitrate reductase, alpha subunit), *narZ* (nitrate reductase), *nxrA* (nitrite oxidoreductase alpha subunit) genes in IASBR 1. A similar trend was observed for the top contributors to the denitrification genes in IASBR 2, except for *nosZ* where *Saprospiraceae*, *Rhodocyclaceae* and *Rhodobacteraceae* families showed equal relative contribution. Overall, the PICRUSt results suggested the metabolic versatility of dominant families contributing to both nitrogen and phosphorus removal genes and the potential impact of influent characteristics on community functional diversity.

3.4 Conclusions

In a recent study our group demonstrated the importance of aeration rate on microbial community structure and the dominance of *Comamonadaceae* members in a laboratory scale IASBR system treating synthetic influent (Gil-Pulido et al., 2018a). In the present study we expand on these investigations via the incorporation of industrial dairy wastewater into the system. Influent levels of NH_4^+-N and PO_4^{3-} -P were found to be key factors shaping bioreactor microbial communities, however IASBR operational parameters applied in this study did not select for traditional nutrient remediation groups. *Comamonadaceae* groups dominated the reactor system under synthetic influent

feeding and it was observed to be one of the most predominant groups during the treatment of industrial influent. *Comamonadaceae* members were found to be key contributors of nitrogen and phosphorus assimilation genes suggesting the potential link of *Comamonadaceae*-related members to biological nitrogen removal. This suggests the importance of conducting further investigations to elucidate the functional link of family members to biological nutrient removal processes, with IASBR systems.

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Chapter 4

Bacterial community composition and nutrient remediation of a pilot-scale IASBR treating dairy wastewater

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Abstract

A pilot-scale intermittently aerated sequencing batch reactor (IASBR) with a working volume of 3000 litres was located at an Irish dairy processing plant and operated for 5 months at a 12 hours cycle length. Each cycle alternated non-aeration (100 mins) and aeration (60 mins) periods to facilitate nitrogen and phosphorus co-removal. The overall bacterial community structure of the IASBR, and its relationship with the influent composition were investigated using Illumina MiSeq sequencing and multivariate redundancy analyses. Under the operational strategy applied, the nutrient removal efficiencies achieved were over 95% for orthophosphate (PO_4-P) and ammonia (NH_4-N). Biological nutrient removal processes rely on the activity of different groups of microorganisms that are related to the stable performance of the system. To date, the overall bacterial community and its potential link to simultaneous nitrogen and phosphorus removal in IASBR is very limited. The current study revealed the bacterial composition of a pilot-scale IASBR was dominated by major phyla such as: *Proteobacteria* (26.5%), *Bacteroidetes* (22.4%), *Planctomycetes* (17.3%), *Armatimonadetes* (11.4%), *Patescibacteria* (7.2%) and *Chloroflexi* (4.9%). Multivariate analyses suggested the correlation of some influent variables analyzed (TSS, PO_4, $PO_4^{—}$P and NO_3^-) with key predominant orders such as *Fimbriimonadales*, *Flavobacteriales*, *Pirelullales* and *Betaproteobacteriales*. Taxonomical analyses performed at pilot-scale were similar to previous reported findings at laboratory-scale operation of IASBR treating industrial wastewater (Chapter 3), where *Proteobacteria*, *Bacteroidetes* phyla and *Betaproteobacteria* class were among the predominant groups of bacteria in the overall community profile of the system. However, the current investigations revealed more complex and diverse ecology in the pilot IASBR system with the presence of bacterial groups that were not previously identified at laboratory-scale such as *Planctomycetales*, *Armatimonadetes* and *Patescibacteria*, during high nutrient performance of the bioreactor. The results suggested that might be other poorly described groups performing nutrient removal in IASBR and contributing to the process stability regardless the community variation.

4.1 Introduction

The dairy industry is experiencing a continuous expansion driven by population growth and the increase in global demand for dairy products (Kothari et al., 2012; Chokshi et al., 2016; OECD, 2018). Global milk production is projected to increase by up to 22% by 2027 compared to the 2015−2017 base period with the European Union (EU) as one of the largest milk producers (OECD, 2018). In Ireland, 1.2 million of litres of milk have been produced in the country between January and March 2019, representing 8.6% increase for same period last year (CSO, 2019). Currently, Ireland is positioned among the top 20 largest producers of milk in the EU (EC, 2019; Ashekuzzaman et al., 2019). The dairy sector contributes in a large proportion to the national economy and the value of Irish dairy exports showed an increase of 19% (€4.02 billions) during 2017 with cheese as the largest dairy export followed by butter (Bord Bia, 2017). Life cycle assessment (LCA) analyses performed on the dairy sector to date have examined the environmental impacts associated with dairy processing industries (EC, 2006; EPA, 2008; Pagan et al., 2010; Geraghty, 2011, Fantin et al., 2012; Finnegan et al., 2015, Finnegan et al., 2018a). Energy demand, water consumption and wastewater generation have been identified as key environmental issues of the dairy sector, as previously detailed in this thesis (see Chapter 1, section 1.3). As a result of the large water consumption in dairy processing plants, the dairy industry generates a vast volume of wastewaters per unit of product processed that must be properly treated prior discharge into receiving water bodies (Brião and Tavares, 2007; Kolev Slavov, 2017). In Ireland, emission levels to water bodies are established by the Environmental Protection Agency (EPA) according to the Best Available Techniques (BAT) for the treatment of dairy processing wastewaters. The accepted limits for Chemical Oxygen Demand (COD), Total ammonia, Total Nitrogen (TN) and Total Phosphorus (TP) are as follows: 15-250, 10, 5-15 and 2-5 mg L^{-1}, respectively (see Chapter 1, Table 5). Typical volumes and composition of wastewater generated in the European dairy industry are detailed in Chapter 1 (see tables 2, 3.1, 3.2 and 4).

Conventional secondary anaerobic and aerobic biological wastewater treatments are the most common technologies for organic and inorganic nutrient removals from dairy processing wastewaters (Britz et al., 2006; Kolev Slavov, 2017). Some examples of typical anaerobic and aerobic treatments are activated sludge, anaerobic digester and upflow anaerobic sludge blanket (see Chapter 1, Table 6). The selection of the most appropriate technology is based on a balance between technical and economic factors, wastewater characteristics and permitted emission limits. In Ireland, the predominant technologies used in dairy processing sites are activated sludge aeration, bio-towers and oxidation ditches (EPA, 2016). The majority of the processing plants combine different systems, such as membrane bioreactor (MBR) and anaerobic digestion system, to achieve better nutrient removals (EPA, 2016). Some of the main disadvantages found in the current available treatments are related to the use of chemicals and the costs associated with treatment plant operations such as for example aeration and the construction and maintenance of different tanks for nutrient removal (EPA, 2016) (see Chapter 1, Figure 9).

SBR has long been considered an optimal treatment option for industrial effluents because of its high degree of process flexibility and stability (Kolarski and Nyhuis, 1997; Britz et al., 2006; Kolev Slavov 2017). Several studies to date have reported SBRs and their different configurations as a good solution for dairy processing wastewater treatment, achieving high organic matter and nutrient removal efficiencies (Tam, 1986; Li and Zhang, 2002; Bae et al., 2003; Sirianuntapiboon et al., 2005; Schwarzenbeck et al., 2005) (see Chapter 1, Table 6). Intermittently Aerated Sequencing Batch Reactors (IASBRs) represent another modification to conventional SBRs which impose multiple intermittent aeration periods during the react phase (see Chapter 1, Figure 12) and they have been developed to efficiently treat various wastewater types (Mota et al., 2005; Li et al., 2008; Zhan et al., 2009; Rodríguez et al., 2011; Pan et al., 2013, 2014; Henry, 2014). For example, Pan and co-workers (2013) investigated simultaneous nitrogen and phosphorus removal in synthetic domestic wastewater using laboratory SBR and IASBR systems. Higher removal efficiencies of total nitrogen (TN) and total phosphorus (TP) were achieved with the application of the IASBR

approach, 90% and 74% respectively, compared with the SBR performance, 79% and 63%, respectively (Pan et al., 2013). In a different study, when IASBR technology was applied to the treatment of slaughterhouse wastewater, removals of over 95% for TN and TP were achieved (Li et al., 2008). IASBR technology has been also recently applied for the treatment of synthetic and industrial dairy processing wastewaters and removal efficiencies of over 90% have been reported for ammonia (NH_4-N) and orthophosphate (PO_4-P), respectively (Leonard 2018a, 2018b; Gil-Pulido et al., 2018; Tarpey, 2016). These preliminary investigations highlighted the potential of the IASBR technology to provide a high efficiency treatment approach for dairy processing wastewater.

The intermittent aeration strategy applied in IASBRs offer some advantages compared to other existing technologies for nutrient removal such as reduction of readily biodegradable COD (rbCOD) demand, long-term stable partial nitrification and favour simultaneous nitrification/denitrification and phosphorus removal in one reactor (Zeng et al., 2003; Orhon et al., 2005; Lit et al., 2008; Li et al., 2011; Henry, 2014). Stable partial nitrification results in a reduced oxygen demand for ammonia conversion and a reduced organic substrate requirement for subsequent denitrification (Li et al., 2011). By minimizing the occurrence of N removal, IASBR technology reduces the demand for rbCOD from denitrifiers and phosphorus accumulating organisms (PAOs) which compete for the carbon source (Li et al., 2008). During the aerobic periods, aerobic nitrifiers oxidize NH_4-N to nitrite (NO^{-2}) and nitrate (NO^{-3}), and during non-aeration periods denitrification occurs. The organic carbon stored by PAOs can then be used by denitrifiers during aerobic periods and nitrogen removal can occur (Orhon et al., 2005; Li et al., 2008). Each nutrient removing micro-organism requires specific growth conditions that need to be considered in the design and operation of IASBRs in order to achieve high removal efficiencies. A satisfactory balance must be achieved to meet the requirements of functional groups involved in wastewater treatment by means of IASBR technology for optimal removal of both nutrients. The limited information available on the overall microbial community structure of IASBR is restricted to previous investigations performed by the group on

laboratory scale systems (Gil-Pulido et al., 2018a, 2018b). These studies were the first to profile the bacterial ecology underpinning an IASBR and revealed the dominance of *Comamonadaceae* related-members during optimal nutrient performance of the bioreactor. In addition, it was demonstrated that the IASBR strategy did not favour the selection of well described/traditional nitrifiers such as *Nitrosomonas* sp. or *Nitrospira* sp. as was previously reported by other studies where nitrifying bacterial populations were investigated in intermittently aerated SBRs (Otawa et al., 2006, Pan et al., 2014).

The study of biological processes at laboratory scale represents the first step in the characterisation/evaluation and optimisation of potential new technologies for scale up. Laboratory scale reactor conditions are designed to mimic, as closely as possible, the real time settings but those conditions rarely resemble full-scale plants (Mielczarek et al., 2013; Crater and Lievense, 2018). From the point of view of the microbiology linked to biological wastewater treatments, it can be challenging to scale-up the bioreactor volume and to deal with a greater number of parameters that affect the structure and dynamics of the system such as the variations in influent composition (Lanham et al., 2013). Molecular studies applied to the investigation of microbial characterization of full-scale plants are of great importance for the optimal operation of those plants but limited investigations have been carried out to date (Mielczareck et al., 2013). Therefore, this work sought to expand the knowledge of bacterial community structure of pilot-scale IASBR during the treatment of dairy processing wastewaters. A number of activated sludge samples collected from a pilot-scale IASBR located at an Irish dairy plant were subjected to 16S high throughput sequencing to characterise composition and transitions in the microbial population over time. Statistical approaches were used to evaluate whether influent characteristics had an impact upon community dynamics.

4.2 Material and Methods

4.2.1 Pilot-scale operation, analytical methods and sampling

The pilot-scale IASBR (Fig. 1) was installed onsite at an Irish dairy processing plant. The IASBR system was placed downstream from a Dissolved Air Flotation (DAF) unit, where aluminium sulphate ($Al_2(SO_4)_3$) was added to precipitate phosphorus (P) prior to secondary treatment. The pilot plant had a working volume of 3000 L, treating 750 L daily. The choice of the applied operational conditions was based on previous laboratory-scale tests carried out at the Environmental Engineering laboratory in the Department of Civil Engineering, National University of Ireland, Galway. A programmable logic controller (PLC) (Siemens LOGO! 8, Germany) controlled pumps and aeration system to set the cycle length, Hydraulic Retention Time (HRT), Solid Retention Time (SRT) and aeration regime. The aeration was supplied in an ON/OFF mode via a compressor (Diaphragm pumps, EL-S serie, 300W) connected to air diffusors located at the bottom of the tank (Leonard et al., 2018b).

The IASBR unit was operated in 12 hour cycles with an intermittent aeration strategy of 8 alternating periods of non-aeration (100 min) and aeration (60 min) per cycle during 5 months (148 days). At the beginning of each cycle, 375 L were pumped into the pilot-scale IASBR, giving the system an HRT of 4 days. A final 80 min period without aeration or mixing was incorporated at the end of the cycles to facilitate sludge settling and effluent decanting, before the next cycle commenced. The system was initially seeded with sludge from an aeration tank at the dairy processing plant where the unit was located. A 93.75 L volume of mixed liquor was removed from the reactor once each day as a sludge waste, resulting in 16 days SRT. (Leonard et al., 2018b). The operational temperature was 20 ± 2°C.

A refrigerated auto-sampler collected effluent samples periodically, which were stored at 4 degrees for further analysis. Influent and effluent samples were analysed periodically by collaborators at NUI Galway, Ireland. Total Organic Carbon (TOC), Total Nitrogen (TN) and Total Phosphorus (TP) were tested using a Biotector TOC, TN, TP Analyser. Ammonia (NH_4-N), nitrite

(NO^{-2}), nitrate (NO^{-3}) and orthophosphate (PO_4-P) were tested using a Konelab 20 Nutrient Analyser (Thermo Scienctific), in accordance with the manufacturer's instructions. Standardized analytical procedures (APHA, 2005) were performed to test Total Suspended Solids (TSS) and Chemical Oxygen Demand (COD) (Leonard et al., 2018a, 2018b).

Figure 1. On-site pilot-scale IASBR unit at an Irish dairy processing plant.

4.2.2 Metagenomic DNA extraction and sequencing

A summary of the samples used for metagenomic and community dynamics analyses is presented in Table 1. Activated sludge samples from within the pilot-scale IASBR tank were collected periodically in sterile bottles and immediately placed at -20 °C until microbial diversity studies were performed at University College Cork, Ireland. A subset of 15 biomass samples was selected for metagenomic analyses and comprised representatives of differing SRTs. To ensure sufficient biomass for optimal nucleic extraction, 6 ml of sludge was centrifuged for 15 minutes at 5000 r.p.m before re-suspending pellets in 1 ml of phosphate buffered saline (PBS). A 300 µl volume of the concentrated biomass was then processed using a PowerSoil DNA Isolation Kit (MOBIO Laboratories) for DNA extraction, according to the manufacturer's instructions. Each sample was processed in triplicate The concentration and purity of the extracted DNA was determined by NanoDrop (ND-1000, Thermo-Fisher, DE, USA) and visualized via 1% agarose gel electrophoresis, SafeView (NBS Biologicals) staining and UV trans-illumination. The samples were submitted for library preparation and sequencing to MACROGEN (Seoul, South Korea). Illumina MiSeq was applied to investigate the bacterial community structure and composition using the primer set 337F (GACTCCTACGGGAGGCWGCAG) and 805R (GACTACCAGGGTATCTAATC) which amplify the hypervariable regions V3-V4 of the bacterial 16S rRNA gene (Takahashi et al., 2014; Fan et al., 2017).

Table 1: Summary of the analyzed activated sludge samples from the full-scale IASBR system. (* Sample 1 represents the initial seed sludge).

Sample ID	Day of operation since starting (day)	SRT period
1*	-	-
2	9	1
3	16	1
4	24	2
5	38	3
6	45	3
7	52	4
8	66	4
9	86	6
10	93	6
11	100	6
12	111	7
14	129	8
15	134	8
16	143	9

4.2.3 Bioinformatics pipeline and taxonomy assignment

The raw paired-end sequences were first quality-screened using DADA2 (Callahan et al., 2016). Paired-end sequences that had any ambiguous base were discarded from the analysis. The remaining sequences were then used to build a parametric sequence error model based on a binomial distribution, and sequences were hierarchically clustered into groups based on the resultsobtained from the error model. The remaining reads were merged into amplicon sequence variants (ASVs), which were further analyzed for quality using Mothur (Schloss et al., 2009). The ASVs were aligned against the SiLVA SEED v132 database through the nearest neighbour algorithm and kmer search method using kmers of 8 bp length under Needleman

conditions. The ASVs that did not start and finish at the position of the forward and reverse primers were removed from the analysis. The remaining ASVs were checked for chimeras using VSEARCH (Rognes et al., 2016) and UCHIME (Edgar et al., 2011), eliminating all chimeric sequences from the analysis. Finally, all remaining ASVs were classified taxonomically against the SiLVA SEED v132 database through the nearest neighbor algorithm and kmer search method with 8 bp length kmers under a taxonomic cut-off of 80%, removing those sequences that did not classify within the domain Bacteria. The high-quality ASVs obtained after the quality control were used to compute a Phylip distance matrix over themselves. The distance matrix was then used to cluster the ASVs into OTUs using the OptiClust algorithm (Schloss, 2016) under a taxonomic similarity of 97%.

A heat map was generated to represent the taxonomy at phylum and order level. The heat maps were based on the relative abundance ≥ 2% in at least one sample and were created with Microsoft Excel.

4.2.4. Statistical analyses

The similarity of the bacterial communities among samples was assessed by the means of Dirichlet multinomial mixing modelling (DMM) (Holmes et al., 2012) and one-way PERMANOVA (Weiss et al., 2017) analyses. For DMM, Operational Taxonomic Unit (OTU) tables at OTU level was used for computation of groupings, from 1 to the whole number of samples, with minimum Laplace's approximation value, considering that the lowest value was the best fit for the groupings model. The one-way PERMANOVA analyses were done using PAST (Hammer et al., 2006) and computed using Bray-Curtis distances and 9999 bootstrap replications. The one-way PERMANOVA analyses were performed to assess the influence of influent characteristics over the OTUs structure and to observe for significant differences in the influent characteristics and OTUs structure between the groups obtained through DMM modelling. An operational parameter was considered statistically robust at a $p\text{-}value \leq 0.05$.

The influence of influent characteristics over the ecological composition at order level of the pilot-scale IASBR was calculated by means of multivariate redundancy analyses (RDA). Analyzed influent characteristics were as follows: total suspended solids (TSS), ammonium (NH_4-N), nitrite (NO^{-2}), nitrate (NO^{-3}), phosphate (PO_4-P) and phosphorus (PO_4). The calculations for RDA were done using CANOCO 4.5 for Windows software, based on 499 unconstrained Monte-Carlo simulations performed under a full permutation model (Ter Braak *et al*., 2002).

4.3 Results and discussion

4.3.1 Nutrient removal efficiency of the IASBR pilot-scale unit

In a previous study published by members of the group, the first 30 days of the pilot-scale IASBR system operation showed overall high nutrient removal efficiencies of above 95% for both orthophosphate (PO_4-P) and ammonia (NH_4-N) (Leonard et al., 2018b). In the initial study of the pilot-scale IASBR performance, Leonard et al. (2018b) reported optimal nutrient treatment of the wastewater maintaining effluent quality below EPA discharge limits (0.5 mg NH_4-N L^{-1}, 0.8 mg PO_4-P L^{-1}) after system stabilization (Leonard et al., 2018b). Percent removal efficiencies of PO_4-P and NH_4-N during the 5-months of operation are presented in Figure 2. Sustained nitrogen and phosphorus removal of above 95% was generally observed and correlated with the results from initial investigations reported by Leonard et al. (2018b). Concentrations of NH_4-N and PO_4-P in the influent varied during the sampling periods, and the average influent concentrations for NH_4-N and PO_4-P were: 26 mg L^{-1} ± 16.5 and 8.7 mg L^{-1} ± 10.3, respectively (Fig. 2).

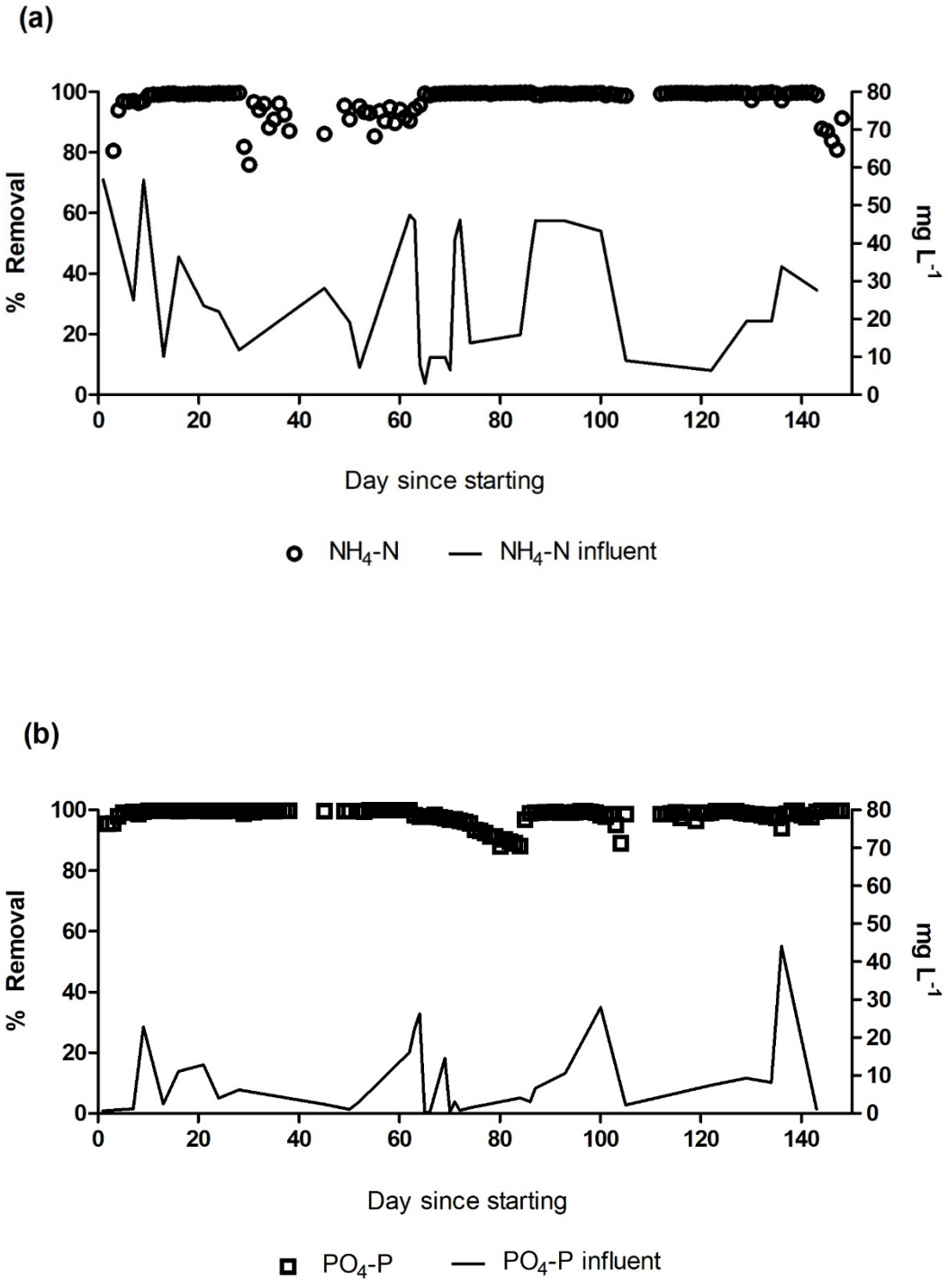


Figure 2. Removal efficiencies (%) achieved the IASBR pilot-scale system where (a) NH_4-N removal efficiencies (%) and NH_4-N content in the influent in mg L^{-1} ; (b) PO_4-P removal efficiencies (%) and PO_4-P content in the influent in mg L^{-1}.

The fluctuating nature of dairy wastewaters has been linked to the type of product processed and it is an important parameter to be considered in the choice of the wastewater treatment (Demirel et al., 2005; Britz et al., 2006). Although the IASBR unit was located following the DAF treatment within the dairy plant, the phosphorus was not consistently removed resulting in fluctuating influent P concentrations entering the IASBR (8.7 ± 10.3 mg PO_4-P L^{-1}). For the case study of the dairy plant where the IASBR was located, the combination of the two processes (DAF and IASBR) led to ~0.1 mg PO_4-P L^{-1} in the effluent for the majority of the days. This demonstrated the potential of IASBR for phosphorus removal as a complement to chemical precipitation in dairy plants. The observed nutrient efficiencies achieved by the full scale system are in line with previously reported NH_4-N and PO_4-P removals from synthetic dairy wastewaters subjected to IASBR treatment (Tarpey, 2016; Leonard et al., 2018a; Gil-Pulido et al., 2018a). The stable and high nutrient performances achieved by the pilot-scale IASBR system during the current study highlighted the potential for application of the technology to nutrient co-remediation and its capacity to successfully treat industrial dairy influents.

4.3.2 Overall bacterial community composition

The bacterial community structure of the pilot-scale IASBR is given in Figures 3 and 4 A limited number of studies on microbial composition of pilot-scale wastewater treatment plants have reported a more complex ecological structure than in laboratory scale bioreactors studies (Wong et al., 2005; Yang et al., 2011; Mielczarek et al., 2013; Lanham et al., 2013). Biological wastewater treatments have shown high complex microbial community profiles that are significantly linked to the overall operational performance of the plant (Yuan and Blackall, 2002; Carvalho et al., 2007; Weissbrodt et al., 2014; Ebrahimi et al., 2010; Wang et al., 2011; Guo et al., 2017; Fan et all., 2017). Therefore, more examples of microbial composition and interactions occurring in pilot and full-scale plants need to be investigated to reveal the mechanisms behind bioreactors performance and to ultimately enhance and optimize biological processes.

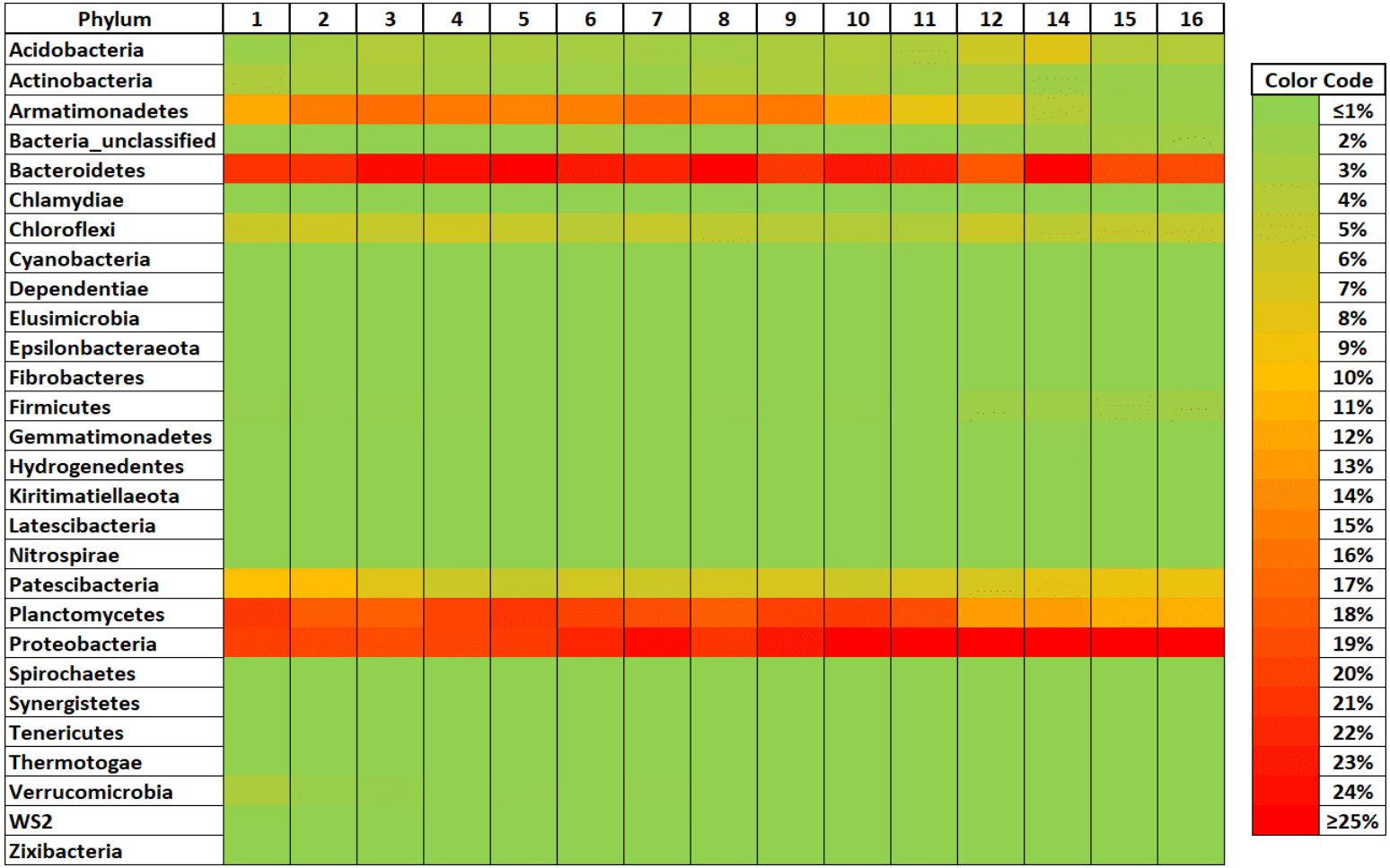
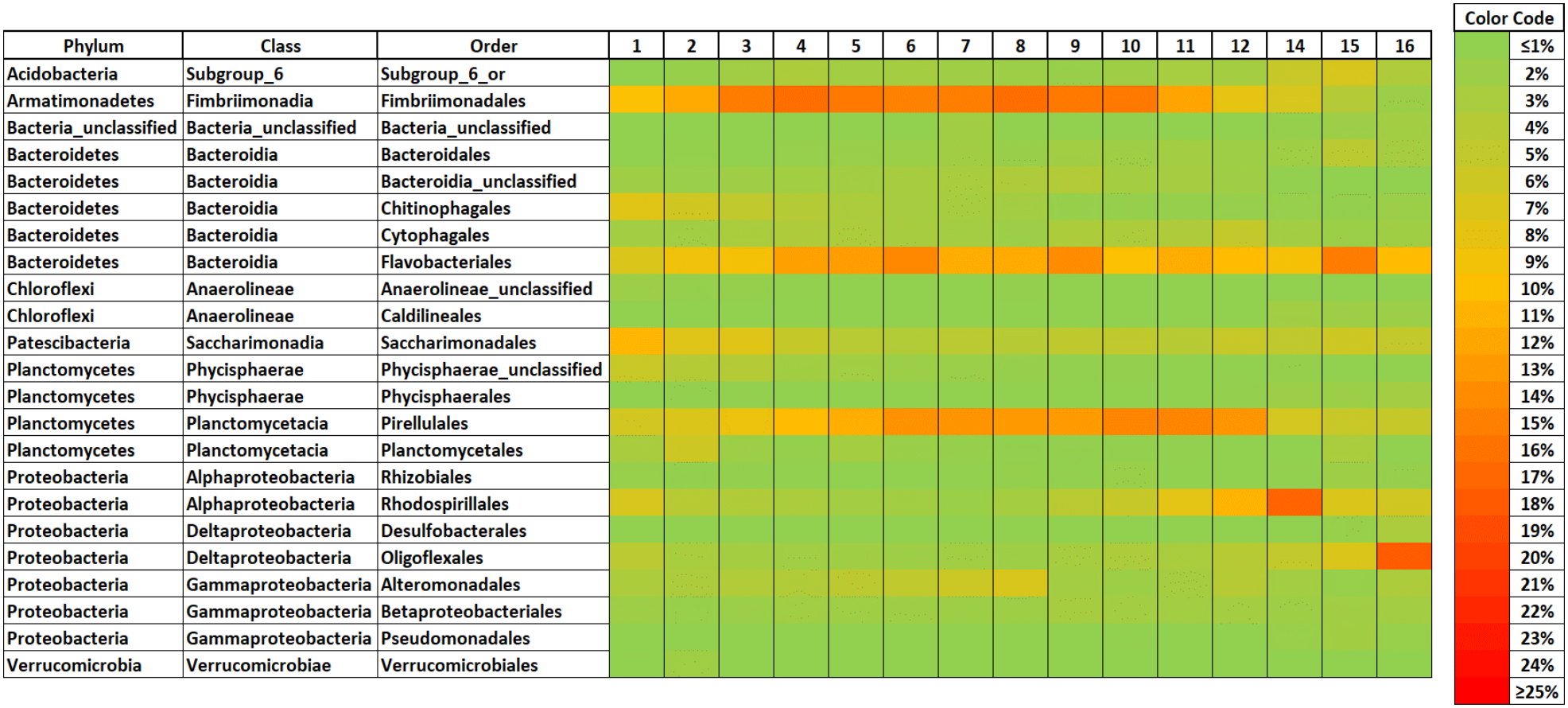


Figure 3. Heat map of the microbial communities in the initial biomass (1) and bioreactor samples (2-16) at phylum level (≥ 2% cut-off applied)



Phylum	Class	Order	1	2	3	4	5	6	7	8	9	10	11	12	14	15	16
Acidobacteria	Subgroup_6	Subgroup_6_or															
Armatimonadetes	Fimbriimonadia	Fimbriimonadales															
Bacteria_unclassified	Bacteria_unclassified	Bacteria_unclassified															
Bacteroidetes	Bacteroidia	Bacteroidales															
Bacteroidetes	Bacteroidia	Bacteroidia_unclassified															
Bacteroidetes	Bacteroidia	Chitinophagales															
Bacteroidetes	Bacteroidia	Cytophagales															
Bacteroidetes	Bacteroidia	Flavobacteriales															
Chloroflexi	Anaerolineae	Anaerolineae_unclassified															
Chloroflexi	Anaerolineae	Caldilineales															
Patescibacteria	Saccharimonadia	Saccharimonadales															
Planctomycetes	Phycisphaerae	Phycisphaerae_unclassified															
Planctomycetes	Phycisphaerae	Phycisphaerales															
Planctomycetes	Planctomycetacia	Pirellulales															
Planctomycetes	Planctomycetacia	Planctomycetales															
Proteobacteria	Alphaproteobacteria	Rhizobiales															
Proteobacteria	Alphaproteobacteria	Rhodospirillales															
Proteobacteria	Deltaproteobacteria	Desulfobacterales															
Proteobacteria	Deltaproteobacteria	Oligoflexales															
Proteobacteria	Gammaproteobacteria	Alteromonadales															
Proteobacteria	Gammaproteobacteria	Betaproteobacteriales															
Proteobacteria	Gammaproteobacteria	Pseudomonadales															
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales															

Figure 4. Heat map of the microbial communities in the initial biomass (1) and bioreactor samples (2-16) at order level (≥ 2% cut-off applied).

Heat map plots at phylum level (Fig. 3) indicated that the bacterial communities in the IASBR were predominated by three main phyla: *Proteobacteria*, *Bacteroidetes* and *Planctomycetes* which accounted for 26.5%, 22.4% and 17.3% relative abundances, respectively. These three phyla, were followed by a few other major phyla with lower relative abundances (<17%) on average: *Chloroflexi* (4.9%), *Patescibacteria* (7.2%) and *Armatimonadetes* (11.4%). In previous studies, *Proteobacteria*, *Bacteroidetes* and *Plactomycetes* phyla were also found to be the most predominant groups in activated sludge communities and in biological nutrient removal processes (Juretschko et al., 2002; Chiellini et al., 2013; Kamika et al., 2014; Weissbrodt et al., 2014; Lawson et al., 2015; Shu et al., 2015; Guo et al., 2017). Guo and co-workers investigated the community diversity and functional profiles of a full-scale simultaneous nitrification-denitrification and phosphorus removal reactor. Their findings revealed the dominance of members belonging to the *Proteobacteria* and *Bacteroidetes* and showed the functional diversity of full-scale wastewater treatment plants compared to laboratory operated bioreactors with more strictly controlled conditions (Guo et al., 2017). Similar profiles for *Proteobacteria* and *Bacteroidetes* observed in the IASBR pilot-scale system were revealed in previous investigations carried out by the group where *Proteobacteria* and *Bacteroidetes* were the most predominant phyla in two laboratory scale IASBRs treating synthetic and industrial dairy wastewater influents (Chapter 2 and Chapter 3). In a study conducted by Lawson and co-workers (2015), *Armatimonadetes* phylum was detected among the bacterial community structure of a pilot-scale EBPR treatment plant (Lawson et al., 2015). Their results reported high activity of *Armatimonadetes* in the EBPR ecosystem irrespective of its low abundance (<1%). Lawson et al. observed that large proportions of rare taxa are potentially active in EBPR environments suggesting that less abundant taxa might also make important contributions to nutrient removal processes (Lawson et al., 2015). Key microorganisms for nitrogen and phosphorus removals described to date are related to the *Actinobacteria*, *Bacteroidetes*, *Nitrospirae, Planctomycetes* and *Proteobacteria* phyla (Wang et al., 2014; Fan et al., 2017; Guo et al., 2017; Marques et al., 2018). Although members of the *Actinobacteria* phylum such

as Candidatus *Accumulibacter phosphatis* (*Accumulibacte*r) and *Tetrasphaera* genera have been identified as to be important PAOs based on their abundances and activity in full-scale EBPR ecosystems (Nielsen et al., 2012), in the current investigations *Actinobacteria* phyla was detected in low relative abundances (<4%) in all the analyzed samples. A similar trend was observed for *Nitrospirae* which was detected at less than 1% relative abundance in the IASBR activated sludge samples.

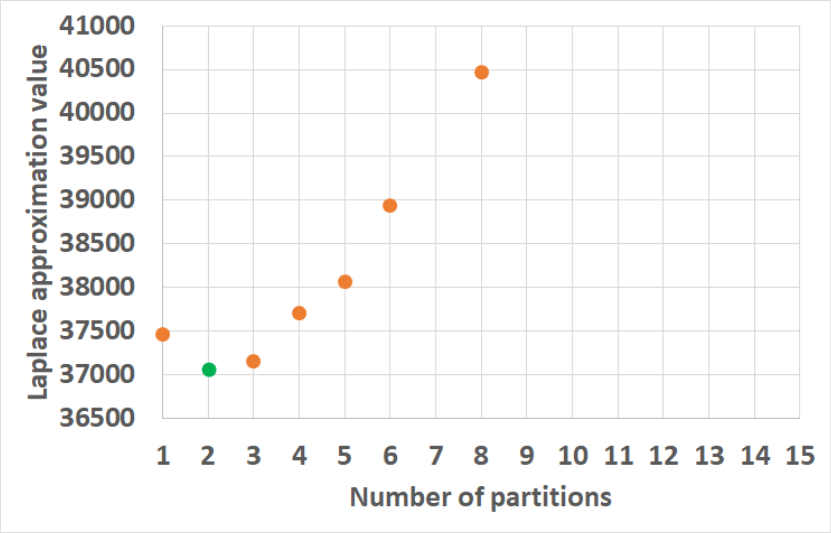
Relative abundances of *Bacteroidetes, Chloroflexi and Patescibacteria* phyla were relative stable across all the samples with 22.4, 4.9 and 7.2% on average, respectively (Fig. 3). The same was not observed for *Proteobacteria* and *Planctomycetes* phyla where variations in relative abundances of the phyla were detected during the 5-month experiment. The abundance of *Proteobacteria* gradually increased to a maximum of 43% at the end of the trial and *Planctomycetes* relative abundance started to decrease from sample 12 to a minimum of 11% on the final day of the experiment. Similar trends were observed for other phyla such as *Armatimonadetes* which decreased relative abundance to limits of 2% by sample 10. Variations in some predominant phyla within the pilot-scale IASBR might be explained by the influence of influent bacterial composition as reported in previous investigations carried out by Lee and co-workers (Lee et al., 2015). In this study, 4 full-scale wastewater treatment plants were investigated over a period of 9 months to determine the impact of influent communities on the temporal dynamics of activated sludge in bioreactors. Their findings showed that regardless of the weak impact of influent wastewater communities, operational taxonomic units (OTUs) detected in activated sludge samples were shared with the influent ecology. Activated sludge communities showed different composition and dynamics to those identified in the influent wastewater. The study concluded that species sorting and bacterial temporal dynamics in activated sludge bioreactors are influenced by influent bacterial communities and ecological effects (Park and Noguera, 2004; Woodcock et al., 2007; Jones and McMahon, 2009; Wells et al., 2014; Lee et al., 2015). Some of the major orders observed within the *Proteobacteria*, *Planctomycetes*, *Bacteroidetes* and *Armatimonadetes* phyla such as *Betaproteobacteriales, Alteromonadales, Planctomycetales, Rhodospirillales,*

Pirellulales and *Fimbriimonadales* (Fig. 4) have been reported in activated and anaerobic sludge samples (Martín et al., 2006; Chamchoi and Nitisoravut, 2007; Sadaie et al., 2007; Liao et al., 2013; Weissbrodt et al., 2014; Third et al., 2005; Shu et al., 2015; Siniscalchi et al., 2017; Sedlacek et al., 2019). Ammonia oxidizing bacteria (AOB) such as *Nitrosomonas* and *Nitrosospira* belong to the *Betaproteobacteriales* order, which are well represented in nitrogen removal systems (van Loosdrecht et al., 2016; Sedlacek et al., 2019). *Flavobacteriale*s and *Planctomycetales* orders were detected as part of the bacterial community of bioreactors performing biological nutrient removal (Third et al., 2005; Martín et al., 2006). Although all anaerobic ammonia oxidixzing bacteria (anammox) identified to date fall into the *Planctomycetales* order (Third et al. 2005) little knowledge exists about the physiological and metabolic role of other members related to the *Planctomycetales* (Chiellini et al., 2013; Guo et al., 2017). To date, the link of the major bacterial groups identified within the community structure of the pilot-scale IASBR to biological nutrient processes is very limited. The microbial composition of the bioreactor suggests the potential role of the described groups in the metabolic and ecological dynamics of IASBRs. Their presence during optimal NH_4-N and PO_4-P bioreactor performance propose the potential link of poorly characterized groups of bacteria to simultaneous nitrogen and phosphorus removal. However, the mechanisms underlying the functional capabilities for the different bacterial groups need to be further investigated.

4.3.3 Analysis of similarity and bacterial correlations with influent characteristics

Similarity of the analyzed activated sludge samples was investigated using Dirichlet multinomial mixing (DMM) modelization and PERMANOVA analysis **(**Fig. 5 and Table 2). DMM method models microbial metagenomics data and provides a suitable probabilistic model to group samples with similar compositions (Holmes et al., 2012; Gu et al., 2017; Rodriguez-Sanchez et al., 2018). According to DMM results (Fig. 5), samples clustered into 2 groups (P1 and P2) at a minimum Laplace approximation value (~37000). These results suggested that during the first 100 days of the pilot-scale IASBR

operation (samples 2 to 11) samples grouped under partition 1 (P1) had a similar type of bacterial community. This also occurred for partition 2 (P2), where samples from day 111 to day 148 (samples 12 to 16) grouped under the same cluster due to their similarity. The factors responsible for driving those changes are however unclear. According to PERMANOVA analyses performed on metacommunities clusters (P1 and P2) and influent concentrations, the analyzed characteristics did not show statistical significance influence ($p > 0.05$) over the bacterial communities in each partition (Table 2b). However, the differences among the OTUs within each group proved to be statistically significant ($p < 0.05$). These results suggested that the change in the community structure of each group is not explained by the influent concentrations of TSS, NH_4^+-N, NO_2^- , NO_3^-, PO_4 and PO_4—P and it might be related to development of the communities across the time.



Sample	P1	P2
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
14		
15		
16		

Figure 5. Results of the Dirichlet multinomial mixing: the left-side graph represent Laplace's approximation value for partition models; table on the right-side shows grouping of samples for the partition models.

PERMANOVA results for partitions 1 and 2 were in accordance with those of the influence of influent concentrations over the overall OTUs structure (Table 2a), where the influent concentrations of the analyzed variables did not show statistically significant influence ($p > 0.05$). The influence of influent composition, bioreactor configuration and operational parameters over the changes in bioreactor microbial structure has been extensively investigated (Zhou et al., 2010; Valentín-Vargas et al., 2012; Gonzalez-Martinez et al., 2016; Chen et al., 2017). These studies have revealed that the influent concentration of some

substrates such as NH_4^+-N, NO_2^- and total nitrogen (TN) drive community variations (Terada et al., 2013; Che et al., 2017). However, other studies have shown weak correlations between microbial community structure and process parameters (Mielczarek et al., 2013; Lawson et al., 2015).

Table 2. PERMANOVA results explaining the influence of influent concentrations over (a) the OTUs structure of the IASBR and (b) the definedmetacommunities (P1 and P2) revealed by DMM modelization.

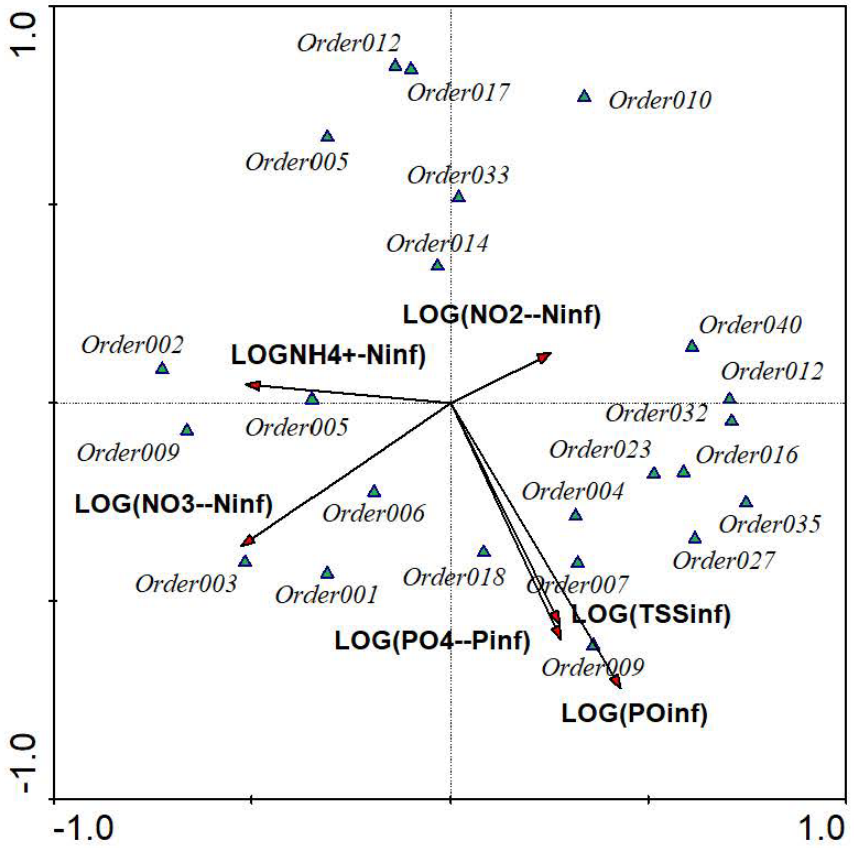
(a)

Variable	F	*p-value*
TSS	1.476	0.2158
NH_4^+-N	0.9891	0.4097
NO_2^-	0.7085	0.6112
NO_3^-	0.9023	0.4226
PO_4	2.704	0.0646
PO_4^--P	2.185	0.1022
OTUs	6.81	0.0016**

(b)

Variable	F	*p-value*
TSS	1.199	0.4021
NH_4^+-N	0.7805	0.6613
NO_2^-	1.457	0.2929
NO_3^-	1.988	0.2838
PO_4	0.481	0.8867
PO_4^--P	0.3326	1

PERMANOVA results presented here suggested that the influent composition did not significantly impact the variations in the community structure of the pilot-scale IASBR, suggesting that other ecological drivers might be involved in bacterial community shaping such as for example bioreactor configuration. An attempt to explain the variance in predominant identified orders was performed via RDA analyses (Fig. 6). Interestingly, variables such as TSS, PO_4, PO_4—P and NO_3^- might explain the observed variance in relative abundance of, for example, the orders *Fimbriimonadales*, *Flavobacteriales*, *Pirelullales*, *Betaproteobacteriales* (Fig. 6). RDA analyses showed that some of the predominant orders have statistical correlation with some of the analyzed variables. According to the length of the arrow represented in the RDA, *Betaproteobacteriales, Bacteroidales, Subgroup_6* are strongly, positively correlated with the content of TSS and phosphorus in the influent and negatively favoured by the ammonia concentration. *Pirelullales* showed high positive correlation to the content of NO_3^- in the influent. Other orders such as *Oligoflexales, Saccharimonadales* and *Chitinophagales* did not show correlation with the studied variables. In summary, while the overall community structure of the pilot-scale IASBR was not impacted from variables analyzed in the current study, community variation at order level indicated that the occurrence of some predominant orders were mostly correlated to variations in investigated variables (TSS, PO_4, PO_4—P and NO_3^-), which did not affect performance of the system.



ORDER CODE	ORDER
Order007	*Subgroup_6_or*
Order002	*Fimbriimonadales*
Order009	*Bacteroidales*
Order017	*Chitinophagales*
Order001	*Flavobacteriales*
Order010	*Saccharimonadales*
Order003	*Pirellulales*
Order012	*Oligoflexales*
Order018	*Betaproteobacteriales*

Figure 6. Multivariate redundancy analysis of the dominant orders against the operational parameters: ammonia (NH_4-N), orthophosphate (PO_4-P), Total Suspended Solids (TSS), Total Phosphorus (PO), nitrate (NO_3^-) and nitrite (NO_2^-) in the influent.

4.4 Conclusions

It has been previously reported that the overall bacterial community structure of laboratory-scale IASBRs during the treatment of dairy processing wastewaters was mostly dominated by the presence of *Comamonadaceae* groups (Gil-Pulido et al., 2018a; Gil-Pulido et al., 2019). The findings presented in the current study demonstrated more diverse ecological structure in a pilot-scale IASBR with dominance of distinct groups of bacteria and relatively stable bacterial community structure. Under the operational parameters applied to the bioreactor, the community variation did not show a significant impact on bioreactor performance which was shown to be stable during the experiment and achieved removal efficiencies over 95% for nitrogen and phosphorus. The taxonomy results suggested ecosystem stability within the bioreactor and adaptation of the microbiology to the IASBR configuration resulting in a stable nutrient bioremediation. In line with previous investigations at laboratory-scale it was also observed that a pilot-scale the IASBR configuration did not favour the proliferation of key functional groups traditionally linked to biological nutrient removal processes. Our observations suggested the potential of poorly described and less well represented bacterial groups being responsible of the optimal nutrient co-removal occurred in the IASBR unit. This research aimed to broaden our knowledge of IASBR community structure in a final pilot-scale settling and to link the bacterial communities identified to biological nutrient removal processes occurring in IASBRs. Nonetheless, the presented preliminary microbial ecology characterization needs to be further investigated by integrating other molecular techniques such as fluorescence *in situ* hybridization (FISH) or gene expression analyses, to reveal the functional link of core groups of bacteria to the stable co-removal of nitrogen and phosphorus via IASBR application. The integration of various molecular techniques could be useful for further research the microbial diversity of IASBR pilot-scale systems, which could lead to establish potential management strategies at full scale implementations.

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Chapter 5

General Conclussions

5.1 General discussion

Irish dairy processors are likely to face rising pressure from various sources to improve wastewater management in an effort to meet environmental standards and more stringent legislation. The current treatments applied to remediate dairy processing wastewaters in Ireland have present several disadvantages related to chemical precipitants use, sludge production and costs associated with plant operations and multistage treatments, among others (EPA, 2016). Therefore, significant research opportunities exist to develop more sustainable and cost-efficient wastewater treatment technologies for application in the dairy sector. Biological treatments are the most recommended and widely used technologies for the treatment of dairy processing wastewaters with different bioreactor configuration designs available to remove biodegradable pollutants present in the wastewater (EPA, 2008; Fraga et al., 2017). A fundamental background knowledge of the microbiological processes linked to such biological wastewater treatments is crucial to enhance the stability of the systems. Thus, it is necessary to fully characterize and to understand the microbial community structure and its dynamics within the bioreactors and to identify key microbial players for the different process types (Wagner and Loy, 2002; Henze et al., 2008). This knowledge could be used to develop new strategies for improving process performance and stability by engineers in wastewater treatment plants. Intermittently Aerated Sequencing Batch Reactor (IASBR) technology represents an enhancement to the conventional Sequencing Batch Reactor (SBR) – lower spatial footprint, single reactor system of co-nutrient remediation, etc. - design that has been successfully applied for nutrient co-remediation of industrial wastewaters (Zhan et al., 2009; Henry, 2014). IASBRs have demonstrated high nutrient removal efficiencies of >90% for nitrogen and phosphorus during the treatment of domestic and slaughterhouse effluents (Li et al., 2008; Pan et al., 2013). IASBR therefore has significant potential for the treatment of dairy processing wastewater remediation that has previously. Nevertheless, while optimal system performance has been reported, microbial structure characterization and its link to biological processes occurring during wastewater treatment via IASBR

have not yet been described. The study of the microbial ecology underpinning IASBR technology has been limited to the study of nitrifer and denitrifier groups of bacteria (Otawa et al., 2006; Pan et al., 2014) with a lack of a full characterization of the mixed bacterial groups performing both nitrogen and phosphorus removal in IASBR (as discussed in **Chapter 1**). The microbial ecology structure of IASBR systems during nitrogen and phosphorus co-removal treating dairy processing wastewaters has been characterized at laboratory and pilot scales under different operational parameters as described in **Chapter 2**, **Chapter 3** and **Chapter 4**.

In **Chapter 2**, sludge from a municipal wastewater treatment plant was used to seed a laboratory-scale IASBR (8 L working volume) subjected to varying aerations (0.4, 0.6 and 0.8 LPM) during the treatment of synthetic dairy wastewater. The most notable observation was the dominance of the family *Comamonadaceae*, within the *Proteobacteria* phyla, during aeration at 0.6 LPM. During that period, *Comamonadaceae* accounted for 82-87% relative abundance in parallel with optimal nutrient removal within the bioreactor (>90% removal efficiencies for NH_4-N and PO_4-P, respectively). Next generation sequencing methodology (454-pyrosequencing) was combined with *in silico* predictive metagenomic analyses with particular focus on bacterial contribution to nitrogen and phosphorus assimilation genes (*nirK, nosZ, norB, ppK, ppX and phbC*). *In silico* predictive metabolic modelling identified *Comamonadaceae* as the major contributor to *ppk, ppx, phaC* and *norB*. The family *Comamonadaceae* has been extensively correlated to biological nutrient removal in particular to denitrification processes (Calderer et al., 2014; Willems, 2014) and more recently to phosphorus removal systems (Ge et al., 2015). The remarkable dominance of *Comamonadaceae* during optimal nutrient co-removal under low aeration and the family contribution to nitrogen and phosphorus genes of interest suggested that *Comamonadaceae* play and important role in both nitrogen and phosphorus bioremediation.

The knowledge of the IASBR ecological profile under conditions of synthetic influent and low aeration was expanded via the incorporation of industrial dairy wastewater into a laboratory-scale IASBR system as presented in **Chapter 3**. Comparison between the population profiles and dynamics of two

laboratory-scale IASBR systems (8 L working volume each) treating synthetic and industrial dairy processing wastewaters revealed the adaptation of the initial biomass (sludge from a full-scale Irish dairy processing plant) to the IASBR configuration with the emergence of a dominance of *Comamonadaceae*-related members. Such dominance was not observed in the original biomass sample. *Comamonadaceae* groups identified such as *Polaromon*as, *Hydrogenophaga* and other *Comamonadaceae* were shown to be impacted by the wastewater type, as defined by NH_4-N and PO_4-P content in the influent. These correlations corresponded to their observed relative abundances shifts and suggested that the occurrence of specific bacterial species in the ecological profiles of IASBR may be shaped by influent levels of NH_4-N and PO_4-P. Additionally, the *in silico* studies confirmed the key contribution of *Comamonadaceae* to phosphorus and nitrogen genes in line with predicted functional studies outlined in Chapter 2.

The overall presence of *Comamonadaceae* family and related-members observed as part of the microbial biomass fraction of IASBRs laboratory-scale set up, suggested that the family *Comamonadaceae* may play a number of important roles in biological nutrient removal processes beyond the evidences reported in the literature to date. The analyses performed in Chapter 2 and Chapter 3 provide theoretical support to the currently emerging profile in the literature of *Comamonadaceae* members as potentially significant contributors to nutrient remediation processes in the wastewater sphere. According to our findings, it would be reasonable to assume that the operational conditions imposed by the laboratory-scale IASBR systems (intermittent aeration) along with the operational parameters applied (low aeration, wastewater type and low temperature) impact the overall bacterial profile underpinning IASBR during nutrient bioremediation. What is more, our investigations conducted at laboratory-scale demonstrated that IASBR technology did not favour the proliferation of well described nitrifiers and PAOs such as *Nitrosomonas* or *Accumulibacter*, respectively, despite optimal nutrient removal performances, and select for similar specific groups of bacteria regardless of the initial biomass composition. Nonetheless, further research needs to be performed (e.g. qPCR,FISH, FISH-DAPI, biopolymer specific staining and gene expression analyses) to

stablish the functional link of described groups of bacteria underpinning IASBR to biological nutrient removal processes within IASBR systems.

Chapter 4 investigated the microbial characterization and population dynamics of a pilot-scale IASBR operated during 5 months and located at an Irish dairy processing plant. The working volume of the onsite system was 3000 L, which gave more accurate indication of its performance at a dairy processing industry, compared to the 8 L of the laboratory-scale systems. Metagenomic analyses of bioreactor samples from the pilot-scale trial based on Illumina Miseq revealed distinct bacterial community profiles compared to the bacterial composition of laboratory-scale IASBR systems, as detailed in the preceding paragraphs. In general terms, the microbial community profile in the pilot-scale IASBR unit showed higher diversity and the presence of dominant phyla identified in the laboratory-scale trials such as *Proteobacteria* and *Bacteroidetes*. The dominance of *Proteobacteria* (26.5%) and *Bacteroidetes* (22.4%) within the system is also well in line with reported findings of the community profile in biological nutrient removal reactors within distinct bioreactor configurations (Kamika et al.2014, Lawson et al., 2015; Guo et al., 2017). Interestingly, other groups not observed in the community structure of the laboratory-scale such as *Planctomycetes* (17.3%), *Patescibacteria* (7.2%) and *Armatimondetes* (11.4%) emerged in the predominant bacterial profiles identified at pilot-scale. Key microorganisms for nitrogen and phosphorus removals described to date are related to the dominant orders observed within the system such as *Betaproteobacteriales*, *Rhodospirillales*, *Plactomycetales* and *Flavobacteriales*, members of the *Proteobacteria, Planctomycetes* and *Bacteroidetes* phyla, respectively (Fan et al., 2017; Guo et al., 2017; Marques et al., 2018). The stability within the bioreactor and the adaptation of the microbiology to the IASBR configuration resulted in an optimal nutrient co-removal (>95%), which was not affected by fluctuating nutrient content in the influent. The presence of dominant bacterial groups during high removal of NH_4-N and PO_4-P suggested their potential link to simultaneous nitrogen and phosphorus bioremediation within the IASBR. However, it is important to take into consideration the presence of a number of other poorly characterized groups within the overall ecological profile of the system. The presence of rare taxa and/or taxa present in low

relative abundances may make important contributions to the BNR process (Lawson et al., 2015) and being potentially active in bioremediation systems. Interestingly, our observations suggest that the IASBR pilot-scale system contain representatives of well-stablished nutrient removal bacteria in addition to less well characterised and present in low abundances, but potentially important organisms for BNR processes. Further exploration of this topic will require more in-depth analysis of community dynamics and the use of other complementary techniques such as fluorescence in situ hybridization (FISH) or quantitative PCR (qPCR) to the tools used in the conducted investigations in order to establish the functional significance of the modelled outputs. The most notable difference between microbial ecology profiles of laboratory-scale and pilot-scale IASBR systems is the low representatives from the *Betaproteobacteria* class in the pilot-scale unit (<1.5% relative abundance), taxonomic classification where *Comamonadaceae* family falls into. We hypothesized that the controlled conditions of low aeration and temperature applied during the laboratory-scale trials may have played a key role in shaping the observed *Comamonadaceae* profile and could explain the differences in the overall ecological picture of the IASBR at pilot-scale. The impact of aeration on the relative abundance of *Comamonadaceae* family has been demonstrated and reported in other studies (Sadaie et al., 2007; Xin et al., 2016) and it is possible that a threshold oxygen concentration provides a selective pressure for *Comamonadaceae*. Temperature is considered one of the operational parameters that greatly affects microbial community structure in wastewater treatment plants (Griffin and Wells, 2017) and some *Comamonadaceae* related members such as *Polaromonas* were identified among the dominant bacterial groups in activated sludge, granular activated carbon (GAC) filters treating wastewaters at low temperatures and within the bacterial community structure of a full-scale wastewater treatment plant in the Polar Artic (Magic-Knezev et al., 2009; He et al., 2016; Gonzalez-Martinez et al., 2018). Further exploration of the impact of aeration and temperature conditions in IASBRs on *Comamonadaceae* related-members might therefore be of interest for future possible applications in the biological wastewater treatments context.

5.1 Concluding remarks

The work presented in the current thesis has conducted first step investigations into the characterization and potential functional link of bacterial groups observed in IASBR systems to co-removal of nitrogen and phosphorus. When the investigations carried out in this thesis commenced, the understanding of bacterial community structure and dynamics of IASBR systems was limited. Overall, our work suggests the metabolic versatility of dominant families contributing to both nitrogen and phosphorus removal within IASBR and the potential impact of operational parameters such as aeration, temperature and influent characteristics. Although the full knowledge of microbial communities' functionality within IASBRs is still not fully understood, our work provides a valuable knowledge of the IASBR bacterial composition during optimal nutrient removal (BNR) performance of the bioreactor. Additionally, the potential link established between the identified communities and BNR processes further enhance the knowledge of key bacterial groups involved in biological nitrogen and phosphorus removal providing new theoretical inputs for future exploitation. However, comprehensive further exploration is required as discussed above, which could lead to optimized biological processes within the system and management strategies for full scale implementations. Continued application of molecular tools in combination with statistical approaches and culture-dependent techniques, will be essential to reveal the functional link of core groups of bacteria to the stable co-removal of nitrogen and phosphorus via IASBR application, to ultimately fully understand rector performance.

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Chapter 6

Additional work

This chapter contains elements of original work presented by (a) Vanessa Horgan Field BSc. for the degree of Microbiolgy (b) Brian Stanley MSc. for the degree in Food Microbiolgy and (c) BSc. Cathal Morrissey for the degree of Microbiology. All investigations were co-designed and supervised by Beatriz Gil Pulido as part of her PhD.

Screening of IASBR activated sludge samples for *Polaromonas* sp. isolates and investigation of *Polaromonas naphthalenviorans* CJ2 type strain for BNR associated functionality.

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Abstract

The dominance of *Polaromonas* sp. within the bacterial community structure of a laboratory-scale IASBR system was observed in this study via molecular-based investigations (Gil-Pulido et al., 2018b). Furthermore, *in silico* predictive metagenomic analyses suggested a high relative contribution from these genera of key functional genes involved in nitrogen and phosphorus metabolic pathways (Gil-Pulido et al., 2018a, 2018b). In this supplementary work, diverse screening approaches were applied to IASBR activated sludge samples from operational periods demonstrating high relative abundance of *Polaromonas* in an attempt to cultivate respective isolates of this genus for functional characterization. Despite the use of published methodologies and the isolation of a range of wastewater treatment associated genera, the isolation of IASBR related *Polaromonas* species was not possible. Therefore, in an effort to expand functional insights on *Polaromonas* in a wastewater treatment context, the type strain *Polaromonas naphthalenivorans* CJ2 was investigated for gene functionalities associated with BNR processes. In addition, the existing knowledge of type strain growth was extended to the study of alternative culture media for culture growth optimization.

Introduction

Polaromonas sp. are Gram-negative bacteria and members of the *Comamonadaceae* family within the *Proteobacteria* phylum (class *Betaproteobacteria*). The genus was proposed by Irgens et al. in 1996 for bacteria from Antarctic marine water samples (Irgens et al., 1996). There are nine species of *Polaromonas* well characterized to date which have been isolated from diverse sources and have shown common characteristics (Choi et al., 2018; Xin et al., 2016; Willems, 2014). Most of the species of *Polaromonas* are capable of growth under psychrophilic conditions (≤ 4° C) but they have shown a broad range of optimal temperature growth (from 1 to 30 °C) (Magic-Knezev et al., 2009; Yagui et al., 2009; Willem, 2014).

Polaromonas species are distributed worldwide and have been mostly isolated from glacier surfaces (Darcy et al., 2011; Gawor et al., 2016). They have also been found in other environments such as coal-tar contaminated sediment (Jeon et al., 2004), tap water (Kämpfer et al., 2006), soil (Sizova and Panikov, 2007; Weon et al., 2008) and the Antarctic ocean (Irgens et al., 1996) (Table 1)

Table 1. Locations and isolation sources of *Polaromonas* species described to date (Adapted from Margesin et al., 2012; Willems, 2014).

Specie	Isolation source	Reference
P. glacialis	Glacier cryoconite	Margesin et al., 2012
P. cryoconiti	Glacier cryoconite	Margesin et al., 2012
P. naphthalenviorans	Coal-tar contaminated sediment	Jeon et al., 2004
P. aquatica	Tap water	Kämpfer et al., 2006
P. jejuensis	Soil	Weon et al., 2008
P. hydrogenivorans	Forest soil Alaska	Sizova and Panikov, 2007
P. vacuolata	Antartic Ocean	Irgens et al., 1996
P. ginsengisoli	Field Soil	Choi et al., 2018
P. eurypsychrophila	Ice core	Xing et al., 2016

Two complete genomic sequences of *Polaromonas* sp. have been published to date (Coleman et al., 2002; Jeon et al., 2004; Mattes et al., 2008; Yagi et al., 2009).*P. naphthalenivorans* CJ2 was isolated from a coal-tar contaminated freshwater sediment (Jeon et al., 2004) and *Polaromonas* Strain JS666 from granular activated carbon filter treating groundwater contaminated with chloroethene (Coleman et al., 2002; Mattes et al., 2008).

These genomic investigations have revealed the significant metabolic capabilities of *Polaromonas* sp. for bioremediation. Most recently, molecular analyses

based on the study of the 16S rRNA have revealed the presence of the genera *Polaromonas* within the microbial community structure of bioreactors (Magic-Knezev et al., 2009; He et al., 2016; Gonzalez-Martinez et al., 2018). He and co-workers identified *Polaromonas* as part of the ecological structure of an activated sludge system treating municipal wastewater at 5°C (He et al., 2016). In the investigations conducted by Magic-Knezev, *Polaromonas* was one of the most frequently observed bacteria in granular activated carbon (GAC) filters operating under varying dissolved organic carbon concentrations (Magic-Knezev et al., 2009). The presence of *Polaromonas* sp. has been also reported by Gil-Pulido and co-workers and potentially associated to biological nutrient removal (BNR) during the treatment of dairy processing wastewater at 11°C (Gil-Pulido et al.,2018b). The strong dominance of *Polaromonas* during the optimal nutrient removal performance in a laboratory-scale intermittently aerated sequencing batch reactor (IASBR) suggested that the genera may contribute significantly to BNR processes (Gil-Pulido et al., 2018a). In IASBR systems, nitrogen and phosphorus removals are accomplished in one single reactor but the knowledge of the microbial ecology underpinning the nutrient co-removal in IASBR is still limited (Otawa et al., 2006; Pan et al., 2014; Gil-Pulido et al., 2018a, 2018b). Evidence from the literature shows a positive correlation between several members of the *Comamonadaceae* family and biological nutrient removal processes (Willems, 2014; Ge et al., 2015; Gil-Pulido et al., 2018a). Preliminary *in silico* investigations have suggested the presence of potential homologues of the key nitrogen and phosphorus metabolism genes in members of *Polaromonas* genus (Pegos et al., 2017; Gil-Pulido et al., 2018a). Such genes include nitrogen fixation proteins such as ammonia monooxygenase (*amoA*), nitrite oxidoreductase (*nxr*), hydroxylamine oxidoreductase (*hao*), polyphosphate kinase (*ppk*) and polyhydroxyalkanoate synthase (phaC) genes (Gil-Pulido et al., 2018a, 2018b; Willems, 2014). To date, there are relatively low number of published studies on *Polaromonas*

sp. (Fig. 1) and further investigations need to be conducted to link the functional characterization of the genera to BNR.

From all the above, the objectives of the investigations carried out in this supplementary work were to (a) screen for *Polaromonas* sp. isolates from IASBR activated sludge samples where molecular analyses investigations revealed predominance of the genera, (b) investigate the type strain *Polaromonas naphthalenviorans CJ2* to determine potential functional association of the genus to nutrient bioremediation.

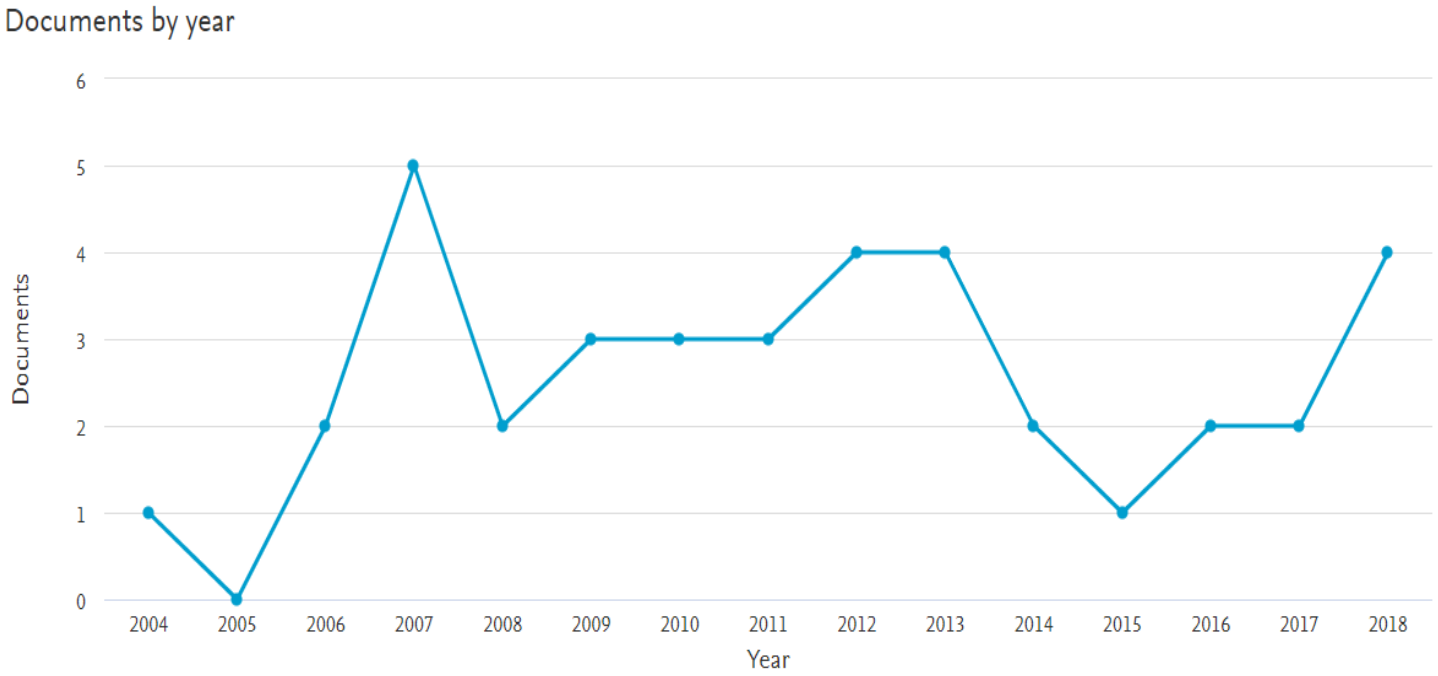

Figure 1. Published articles on Polaromonas spp. from 2004 to date. Data retrieved from Scopus.

Material and Methods

Screening of IASBR activated sludge for *Polaromonas* species

Bacteria were isolated from activated sludge of a laboratory intermittently aerated sequencing batch reactor (IASBR) treating dairy synthetic wastewater at 11°C (Gil-Pulido et al., 2018b). The IASBR strategy imposes non-aeration and aeration periods during the operational cycle to facilitate nitrogen and phosphorus co-removals. Samples used in the current investigations were taken during the third aeration period. The IASBR was operated under varying aeration conditions as described by Gil-Pulido et al. (2018b). The sample choice was based on previous reported metagenomic results where *Polaromonas* dominated the ecological structure of the bioreactor during optimal nutrient performance (Fig.2) (Gil-Pulido et al., 2018b). Samples were collected from the bioreactor operated at NUIG Galway and subsequently frozen (-80°C) before processing for microbiological analyses.

Activated sludge samples were allowed to defrost overnight at 4°C. Defrosted material was then gently mix by inversion. Bacterial isolations were run in triplicate and attempted using different media and approaches:

(a) 100µl of the suspension was spread-plated on R2A (Reasoner's 2A agar) and LB (Luria-Bertani) media. R2A is a low nutrient agar culture medium used in the study of bacteria present in potable water (Sandle et al., 2014) and it has previously been used for the isolation of *Polaromonas* sp. (Magic-Knezev et al., 2009; Xing et al., 2016). Composition is shown on **Table 2**. LB medium (2% m/v) consists of peptone, yeast extract, sodium chloride, beef extract and agar (1%)

(Jeon et al., 2004). Plates were incubated at four temperatures (4°C, 10°C, 21°C and 28°C) according to different optimal growth temperatures reported for species of *Polaromonas* (Willems, 2014) over a period of 6 weeks (Gawor et al., 2016; Ciok et al., 2018). Isolates were recovered at different times during the incubation and subsequently subjected to analyses based on 16S rRNA.

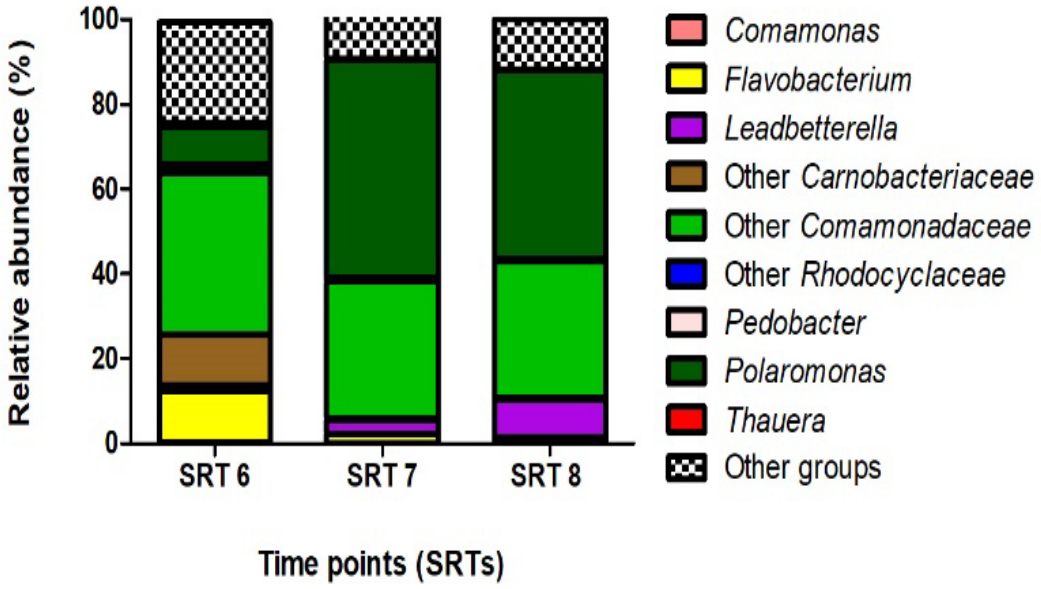

Figure 2. Bacterial community structure of IASBR activated sludge samples from Solid Retention Times (SRTs) 6, 7 and 8 during optimal nutrient performance (%) as presented in Gil-Pulido et al., 2018b.

(b) Serial dilutions on the suspension from 10^{-1} to 10^{-6} were completed and 50µl of each dilution were spread-plated on R3A agar. R3A agar was designed for the sub-cultivation of microorganisms that haven been isolated on R2A (Sandle, 2014) and its formulation is richer in nutrients compared to R2A (**Table 2**). R3A agar plates were then incubated at 4°C in the dark for 6 weeks as previously published (Gawor et al., 2016; Ciok et al., 2018). Additionally, 50µl of the undiluted suspensions were spread-pated on synthetic wastewater (SWW) media (**Table 3**). The composition of the SWW media intended

to reproduce the media conditions of the dairy synthetic wastewater used in the laboratory-scale IASBR investigations were *Polaromonas* sp. was detected to be predominant during optimal nutrient removal (Gil-Pulido et al., 2018b). SWW plates were then incubated under the same conditions of R3A plates. These isolates displayed colony morphologies and growth profiles previously observed in R2A and R3A plates (data not shown). As a result of these similarities and project time limitations, these were not subjected to further molecular characterisation, although representatives were stored at -80°C.

From each plate, at least three of the most abundant colony types with distinctive morphologies were picked when possible. As required, plates were re-streaked until a pure culture was observed prior to single colony picking. DNA isolation was performed by using boiling lysis method (Gawor et al., 2016). Single colonies were picked using sterile tips and suspended in 50µl of sterile 1X TE buffer (10mM Tris, 1mM EDTA). The suspension was then boiled at 98°C for 10 min and subsequently centrifuged to precipitate cell debris. The lysate was then subjected to gel electrophoresis to check quality of the metagenomic extracted DNA. Those colonies that were not further processes were storage at -20°C.

DNA extracts were used as a template for PCR amplification of the 16S rRNA gene using a pair of universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') which amplify the full length of the 16S rRNA gene (Lane, 1991). The reaction mixture (50µl) contained 10X dreamTaq buffer, 2mM dNTPs, 5U/µl dreamTaq DNA Polymerase, 10uM primer forward and primer reverse, 1-10 ng/µl template DNA, Dimethyl sulfoxide (DMSO), Bovine Serum Albumin (BSA) and sterile distilled water to complete the 50µl final volume. A program of initial denaturation at 94°C for 3 min, followed by 29 more cycles of 1 min denaturing at 95°C, 1 min annealing at 50-53°C, 1 min of extension at 72°C, with a final extension step of 72°C held for 5 min. Products in the expected size range (~1500 bp) were then purified using QIAGEN kit (QIAquick PCR Purification Kit, Manchester

UK) before sending to GATC Biotech for Sanger Sequencing using Super Run Service.

Raw sequences reads were viewed and manually edited using the free software FinchTV v1.4 (http://geospiza.com/finchtv, Geospiza Inc., Seattle, WA, USA). Edited sequences were then aligned using Basic Local Alignment Search Tool (BLAST) to find regions of similarity between sequences.

Table 2. R2A and R3A media composition. R3A media formulation was prepared according to Sandle et al. (2014). Final pH 7.2 (± 0.1).

Compound	**R2A (g/L)**	**R3A (g/L)**
Yeast extract	0.5	1.0
Peptone	0.5	0.5
Casamino Acid	0.5	1.0
Glucose	0.5	1.0
Soluble Starch	0.5	1.0
Na-Pyruvate	0.3	0.6
K_2HPO_4	0.3	0.6
$MgSO_4$ X $7H_20$	0.05	0.1
Agar	15	15

Table 3. Synthetic wastewater (SWW) medium composition. Formulation was prepared as described in Gil-Pulido et al. (2018a). Final pH 7.2 (± 0.1)

Compound	Quantity (g/L)
Sodium acetate	2.9 g
Yeast extract	0.218
Dried milk	0.872
NH_4CL	0.1673
Urea	0.1299
Na_2PO_4H	0.126
K_2HPO_4	0.062
$NaHCO_3$	0.13
$MgSO_4 * 7H_2O$	0.05
$FeSO_4 * 7H_2O$	0.01
$MnSO_4 *H_2O$	0.002
$CaCl_2 * 2H_2O$	0.002
Agar	15

Tests on *Polaromonas naphthalenivorans* CJ2

Polaromonas naphthalenviorans CJ2 type strain (DSMZ 15660) (Jeon et al., 2004) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and recovered on DSMZ recommended media. Several stocks of the strain were preserved in glycerol (25%) and stored at -80°C for further analyses.

Polaromonas DSMZ 15660 growth was investigated on R2A, R3A and modified R3A (mR3A) media. In mR3A, glucose was replaced with lactose (1.0 g/L) as a sole carbon source to compare the lactose and glucose utilization. Each media was carried out in duplicate and the growth of the type strain was measured by optical density (OD) at 600nm wavelength (OD_{600}). 5 ml of an overnight *Polaromonas* DSMZ 15660 were added to conical flasks with 20 ml each of R2A, R3A and mR3A, respectively, and incubated at 23°C and 180rpm. The overnight culture was prepared from a fresh colony grown on R3A media. *Polaromonas* DSMZ 15660 growth was monitored on R2A,

R3A and SWW media for 48 hours. At the end of the growth curve experiment, a graph of time in minutes versus OD_{600} readings was plotted to obtain growth curve of type strain monitored during 24 hours. Quantitative determination of lactose and glucose utilisation during growth was performed via high performance liquid chromatography (HPLC).

Polyphosphate (Poly-P) kinase activity was investigated by an adaptation of the acid-shock strategy of Mullan and co-workers, (Mullan et al., 2002) which involved lowering the pH for the R3A media from 7.2 to 5.5. The pH in the R3A media was altered by adding 200µl of 99% hydrochloric acid (HCL). Flasks containing R3A pH 7.2 (control) and R3A pH 5.5 were incubated at 23°C and 180rpm for 4 hours. Bacterial cells were then subjected to 4'6-diamidino-2phenylindole (DAPI) staining to determine the presence of intracellular Poly-P granules under acid stress. DAPI working solution was prepared as described by van Loosdrecht et al. (2016). Samples were first fixed on a glass slide and subsequently embedded with 300µl of DAPI working solution (1µg mL^{-1}). The stain was then allowed to migrate on the sample for 20 min at room temperature protected from direct exposure to light. After the incubation time, the excess of DAPI was removed and rinsed using distilled water (dH_2O) to remove fluorescence background (van Loodsdrecht et al., 2016). Samples were examined under the microscope following air-drying using a Leica fluorescence microscope, and Olympus camera, with a N2.1 filter cube with excitation and emission wavelengths of 358 nm and 461nm, respectively.

The induction of polyhydroxyalkanoate (PHA) accumulation in *Polaromonas* was also evaluated using inorganic nutrient limitation as a stressor by limiting the phosphorus content on R3A (Wen et al., 2010). Dipotassium phosphate (K_2HPO_4) is the source of phosphorous in R3A media and was lowered from 0.6 g L^{-1} to 0.2g L^{-1}. A conical flask with R3A and limiting phosphorus content (0.2 g L^{-1}) was inoculated with *Polaromonas* DSMZ 15660 and incubated at 23°C and 180rpm for 4 hours. The sample was then fixed onto a glass slide and subjected to Nile Blue stain (van Loosdrecht et al., 2016). Reagents used for PHA staining were aqueous solution of Nile Blue (1% v/v) and acetic acid (8% v/v). The slide was dipped into a suspension of 1% aqueous solution of Nile Blue and heated at 55°C for 10 min. The excess of stain was

removed by rinsing the slide with dH_2O at room temperature prior to destaining of with acetic acid (8%) for 1 min. After the incubation time, the slide was washed with dH_2O and allowed to dry at room temperature (van Loosdrecht et al., 2016). The slide was examined under the fluorescence microscope as per DAPI sample settings above.

Predictive functional metabolic modelling on IASBR activated sludge samples

Based on the 16S rRNA sequences investigated on the study presented in Appendix 1, the functional potential of the microbial communities in the bioreactor was predicted using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) approach (Langille et al., 2013). Metagenome contributions were computed in PICRUSt for the prediction of the top contributors for target genes of interest. Full details of the methodology used for *in silico* predictive metagenomics have been presented in Chapter 2.

Results and discussion

Bacterial isolates from IASBR activated sludge

A total of forty-one strains were isolated from IASBR activated sludge samples using R2A, LB and R3A media and different temperatures (Tables 4, 5 and 6). The strains were classified based on the 16S rRNA gene sequence assigned by BLAST with a percentage identity over 97%. Isolation methods in combination with molecular-based analyses are important in supporting the identification and characterization of key bacteria responsible for biological wastewater treatments (Serafim et al., 2002; Ferrera et al., 2016). Culture-dependent methods in combination with molecular technologies are necessary to fully-understand and profile functional capabilities of microbial consortia responsible for wastewater treatments (Narayanasamy et al., 2015; Ferrera et al., 2016).

IASBR activated sludge samples were from the same bioreactor samples analyzed using molecular-based methods previously in this project (Gil-Pulido et al., 2018b). In these investigations, the dominance of *Polaromonas spp*. was observed in the overall bacterial community structure of the bioreactor and particularly during optimal nutrient removal periods (Gil-Pulido et al., 2018b). To date, there is no highly selective procedure reported for the isolation of *Polaromonas* sp. and the available literature is very limited (Willemns, 2014). Isolates of *Polaromonas* sp. have been mainly recovered from glacier samples (Table 1), i.e. highly selective and therefore niche colonised/low diversity environs. Wastewater samples may therefore be particularly challenging for the isolation of the genus given the complex and high diversity mixed cultures systems presented by activated sludge samples. Most of the isolated *Polaromonas* strains to date have been achieved under cold growth temperature and using R2A media (Willems et al., 2014). Over a six-week period, the heterotrophic growth of different microorganisms was monitored in R2A and LB media under 4 different temperatures (Tables 4 and 5). The most abundant strains cultured at 4°C were *Pseudomonas fluorescens* and *Pseudomonas fragi* in both R2A and LB media. As shown in Table 4 and Table 5, diversity of microorganisms was greater in LB than R2A and differences on isolated bacteria were observed to occur depending on the growth temperature. From the strains isolated in LB, R2A and R3A media, only *Flavobacterium* was among the predominant detected species of the IASBR bacterial community structure by molecular-based methods (Gil-Pulido et al., 2018b). *Flavobacteirum* sp. has been linked to biological nutrient removal processes (Weissbrodt et al., 2014) and previously isolated in activated sludge systems (Park et al., 2007; Liu et al., 2010). Other strains isolated such as *Carnobacterium* sp., *Sphingobacter*, *Microbacterium* and *Serratia* have been identified in different wastewater treatments including activated sludge processes (Matsuyama et al., 1999; Sun et al., 2013; You et al., 2007; Gong et al., 2008).

Table 4. Summary of the isolated strains in R2A media. The taxonomical classification at class level for each of the isolates is represented in brackets following the code: 1 = *Gammaproteobacteria*; 2 = *Sphingobacteria*; 3 = *Flavobacteriia*

Isolate id.	BLAST hit	Temperature
1	*Pseudomonas fluorescens* (1)	4°C
3	*Pseudomonas fragi* (1)	4°C
6	*Pseudomonas fragi* (1)	4°C
7	*Pseudomonas fragi* (1)	4°C
10	*Pseudomonas* psychrophila (1)	4°C
11	*Pseudomonas fragi* (1)	4°C
12	*Pseudomonas fragi* (1)	4°C
15	*Pseudomonas psychrophila* (1)	4°C
19	*Sphingobacter* (2)	12°C
20	*Sphingobacter* (2)	12°C
21	*Flavobacterium* (3)	21°C
27	*Sphingobacterium* (2)	28°C

Table 5. Summary of the isolated strains in LB media. The taxonomical classification at class level for each of the isolates is represented in brackets following the code: 1 = *Gammaproteobacteria*; 2 = *Sphingobacteria*; 3 = *Actinobacteria*.

Isolate id.	BLAST hit	Temperature
2	*Pseudomonas fragi* (1)	4°C
4	*Pseudomonas fragi* (1)	4°C
5	*Pseudomonas fragi* (1)	4°C
8	*Pseudomonas fragi* (1)	4°C
9	*Pseudomonas fragi* (1)	4°C
13	*Pseudomonas psychrophila* (1)	4°C
14	*Pseudomonas psychrophila* (1)	4°C
16	*Citrobacter gielenii* (1)	12°C
17	*Citrobacter gielenii* (1)	12°C
18	*Acinetobacter bohemicus* (1)	12°C
22	*Sphingobacterium* (2)	21°C
23	*Shewanella putrefaciens* (1)	21°C
24	*Acinetobacter haemolyticus* (1)	21°C
25	*Acinetobacter hamolyticus* (1)	21°C
26	*Leucobacter salsicus* (3)	21°C
28	*Kluyvera spp.* (1)	28°C

Table 6. Summary of the isolated strains in R3A media at 4°C. The taxonomical classification at class level for each of the isolates is represented in brackets following the code: 1 = *Actinobacteria* 2 = *Bacilli*; 3 = *Gammaproteobacteria*; 4 = *Flavobacteriia*

Isolate id.	BLAST hit
A	*Pseudoclavibacter sp.* (1)
B	*Microbacterium sp.* (1)
C	*Microbacterium sp.* (1)
D	*Trichococcus pasteurii* (2)
E	*Serratia sp.* (3)
F	*Carnobacterium sp.* (2)
G	*Pantoea ananatis* (3)
H	*Flavobacterium* (4)
I	*Trichococcus paludicola* (2)
J	*Leuconostoc lactis* (2)
K	*Stenotrophomonas sp.* (2)
L	*Carnobacterium sp.* (2)
M	*Trichococcus paludicola* (2)

The retrieved results from the isolates detailed in Tables 4, 5 and 6 shown that the screen was broad in so far as it captured representatives from five classes (Gammaproteobacteria, Actinobacteria, Bacilli, Flavobacteriia and *Sphingobacteria*), but interestingly there were no isolates from the *Betaproteobacteria* class, despite the association of so many members of this class with biological wastewater treatments. In summary, the results of this supplementary screening suggest a limitation of published culture-dependent techniques to isolate *Polaromonas* from complex samples with high bacterial diversity. The apparent dominance of *Polaromonas*, as determined by molecular profiling, did not have any positive influence in this regard. Indeed, the absence among the screens of representative isolates

from the Beta-Proteobacteria was notable given the association of same with biological treatment systems.

Significant further investigation is necessary therefore to design a more selective media for *Polaromonas* sp., which may materialise as additional genome information of genus member isolates from diverse environs becomes available for metabolic profiling.

Potential role of *Polaromonas* sp. in biological phosphorus removal

Polaromonas naphthalenivorans CJ2 is one of the two complete genomes of *Polaromonas* strains available and further investigated to date (Jeon et al., 2004; Ciok et al., 2018). The ability of *Polaromonas* sp. to degrade pollutants reportedly includes bioremediation of hydrocarbons and chlorinated ethenes (Mattes et al., 2008; Ciok et al., 2018) but its bioremediation potential in other contaminated environment such wastewaters has not been elucidated yet. Previous *in silico* predictive metagenomic analyses performed on IASBR activated sludge samples by the group, revealed the genus *Polaromonas* as one of the top key contributor of phosphorus assimilation/storage pathway genes such as exopolyphosphatase (*ppx*) and polyphosphate kinase (*ppk*) (Table 7).

Table 7. Correlation of taxonomy and relative contributions to genes of interest: *ppk* and *ppx*.

Taxonomic classification	Contribution (%)	
	ppx	**ppk**
o__Burkholderiales_f__Comamonadaceae_g__	12.5	12.4
o__Burkholderiales_f__Comamonadaceae_g__Limnohabitans	4.2	4.2
o__Rhodocyclales_f__Rhodocyclaceae_g__	4.3	4.0
o__Flavobacteriales_f__Flavobacteriaceae_g__Flavobacterium	7.4	7.4
o__Burkholderiales_f__Comamonadaceae_g__Polaromonas	3.2	3.2

Additionally, data retrieved from the National Centre for Biotechnology Information (NCBI) GenBank, founded 47 homologs in the complete genome of *Polaromonas naphthalenivorans* (accession number NC_008781) related to the nitrogen cycle and phosphorus removal. Most of the identified homologs were related to nitrate reductase genes while the percent identities for *ppk* gene was less consistent. Two experiments were performed on *Polaromonas* DSMZ 15660 type strain in order to assess the functional link of *Polaromonas* sp. to biological phosphorus removal. Intracellular polymers stored by PAOs include polyphosphate (poly-P) and polyhydroxyalkanoates (PHAs) (Serafim et al., 2002). Staining procedures for qualitative visualization of polymers by light microscopy coupled with specific stains have been previously used to detect their presence/absence in polyphosphate accumulating organisms (PAOs) (Serfim et al., 2002; Wen et al., 2010; Tarayre et al., 2016). Techniques for the isolation of PAOs are mainly based on the detection of both intracellular polymers, poly-P and PHA (Tarayre et al., 2016). For the detection of poly-P accumulation, the staining methods commonly used are Neisser, Loeffler's Methylene Blue and DAPI (Serafim et al., 2002; van Loosdrecht et al., 2016). In the optical microscopy visualization of PHA storage polimers, Nile Blue and Sudan Black stains are normally used (Mesquita et al., 2015; Tarayre et al., 2016). The type strain was subjected to two stress tests that have previously been linked with polymeric inclusion formation, namely acid-shock (pH 5.5) and inorganic (N, P or S) nutrient limitation, respectively. These stressors can potentially be used therefore to demonstrate functional capacities for the accumulation of poly-p and PHA on *Polaromonas* DSMZ 15660 cells. Stained cells of *Polaromonas* DSMZ 15660 with DAPI were viewed under the microscope after 4 hours' incubation on R3A pH 7.2 and R3A pH 5.5 to compare poly-P accumulation under the two different media. DAPI staining results indicated the strong presence of poly-P granules in the cells grown under pH 5.5, while it was not observed on those grown at pH 7.2 (Fig. 3). The intracellular phosphorus accumulation at pH 5.5 was observed on both individual and clustered cells (Fig. 3). Results of the effect of phosphorus limitation on the production and accumulation of PHA are shown in Figure 4. According to the visualization of the Nile Blue stain under the microscope, cells grown in the reduced

phosphate media showed PHA accumulation in both individual and clustered cells (Fig. 4a). This was not observed in the original media without phosphate limitation where staining results evidenced less PHA production and only in clustered cells (Fig. 4b).

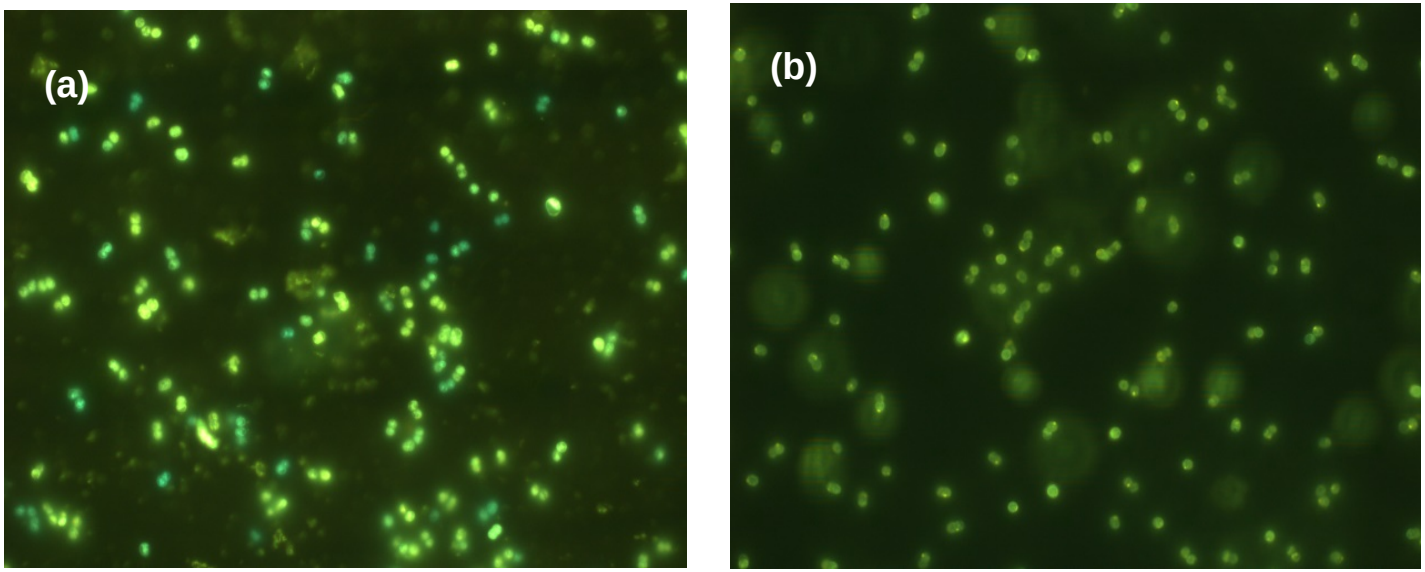

Figure 3. DAPI stained cells for the detection of poly-P (evidenced by green fluorescence) in (**a**) strain grown on R3A pH 5.5 (**b**) strain grown on R3A pH 7.2.

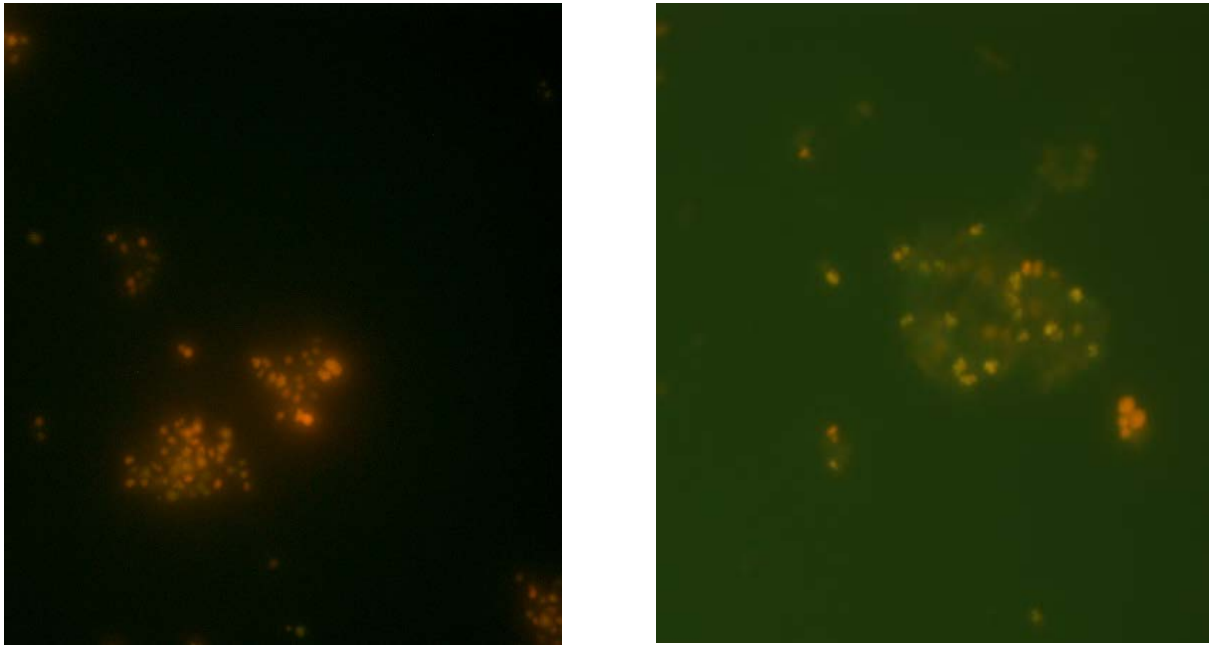

Figure 4. Nile Blue stained cells for the detection of polyhydroxyalkanoate (PHA), evidenced by red fluorescence) accumulation under phosphorus limitation conditions **(a)** and original media without modifications **(b)**.

Staining procedures used to visualize the intracellular polymers storage in *Polaromonas* DSMZ 15660 revealed that the type strain is capable of accumulating Poly-P and PHA under the growth conditions investigated in this chapter. These tests provided further evidence of the functional link to the predicted *in silico* phosphorus genes. Further work is required to identify whether specific genes only are only being transcribed which would require different approaches such as reverse transcription polymerase chain reaction (RT-PCR) on RNA isolations from representative samples. The results presented in this chapter also identified acid-shock as a valuable stressor to determine poly-P accumulation capacities of *Polaromonas* DSMZ 15660 suggesting its potential application in the functional characterisation of other *Polaromonas* strains.

Polaromonas DSMZ 15660 growth in three different media and carbon source utilization

Pure bacterial cultures have been applied to bioaugmentation in wastewater treatment performances with successful applications reported even in full-scale treatment plants (Stephenson, D. and Stephenson, T, 1992; Herrero and Stuckey, 2015; Tang and Chen, 2015). Bioaugmentation with key microorganisms involved in biological nutrient removal, such as nitrifiers, can help during biological process deteriorations in wastewater plants (Tang and Chen, 2015). The cultivation of microorganisms to reinforce biological wastewater treatment populations requires a good knowledge of optimal bacterial growth conditions and/or bacterial consortia preparation (Herrero and Stuckey, 2015). In this study, it was hoped that growth characterisation and bioaugmentation work would be performed with IASBR isolates of *Polaromonas*. However, as reported above, it was not possible to generate same using previously published isolation techniques. The type strain was therefore utilised in a series of growth characterisation experiments to compare recommended growth media and conditions. In order to give new insights into the growth optimization of *Polaromonas* DSMZ 15660, three different media were investigated for culture preparation. R2A has been the

most commonly used medium for the growth and isolation of *Polaromonas* spp. (Willems et al., 2014). More recently, R3A medium has been designed for the sub-cultivation of microorganisms that have been isolated and are capable to grow on R2A (Sandle, 2014). R3A medium is nutritionally richer than R2A (Table 2). A third medium, modified R3A (mR3A), was investigated where glucose was replaced by lactose as a carbon source using same proportions. Lactose utilisation was investigated as it reflects the primary carbohydrate found in dairy processing wastewater (Omil et al., 2003). Growth curve results for each of the three media are presented in Figure 5.

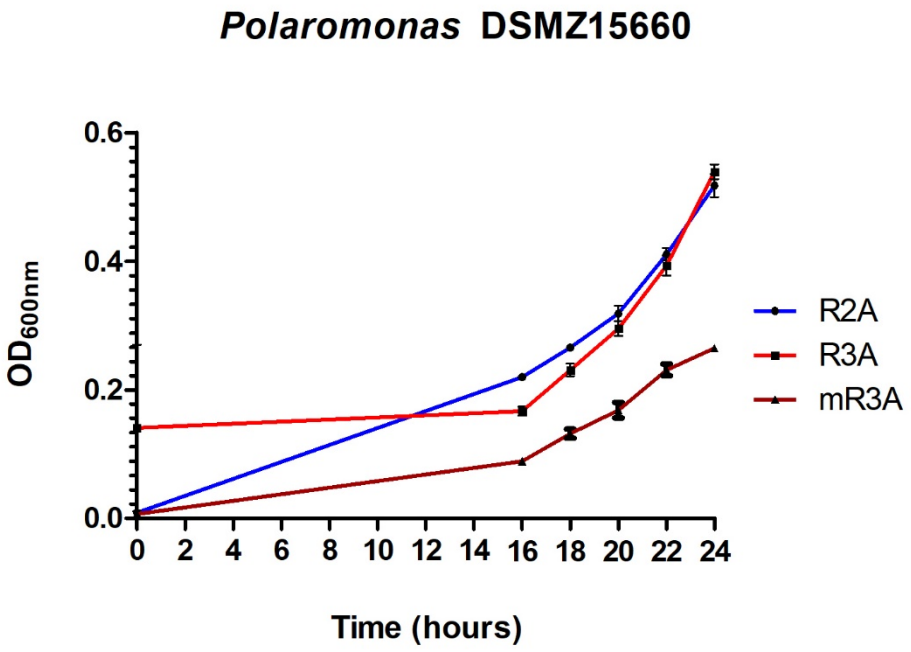

Figure 5. *Polaromonas* DSMZ growth curves on R2A, R3A and mR3A media.

It was observed that for the three media studied, bacterial cells enter in the log phase after ~16 hours of incubation. Growth on R2A and R3A media showed relatively closed behaviour which suggested that R3A media is a suitable alternative media for the growth of *Polaromonas* DSMZ 15660. When the glucose was replaced by lactose in mR3A medium, 50% less growth was observed compared to R2A and R3A media. HPLC results for R3A revealed

lactose removal over time, presumably via uptake/and or lysis (data not shown). Analysis of the KEGG pathway database for associated galactose metabolism genes in *P. naphthalenivorans* revealed an incomplete capacity within the type strain for galactose uptake and utilisation either via the Leloir or phosphoenpyruvate (PEP) transport systems (Figure 6) (Solopova et al, 2018).

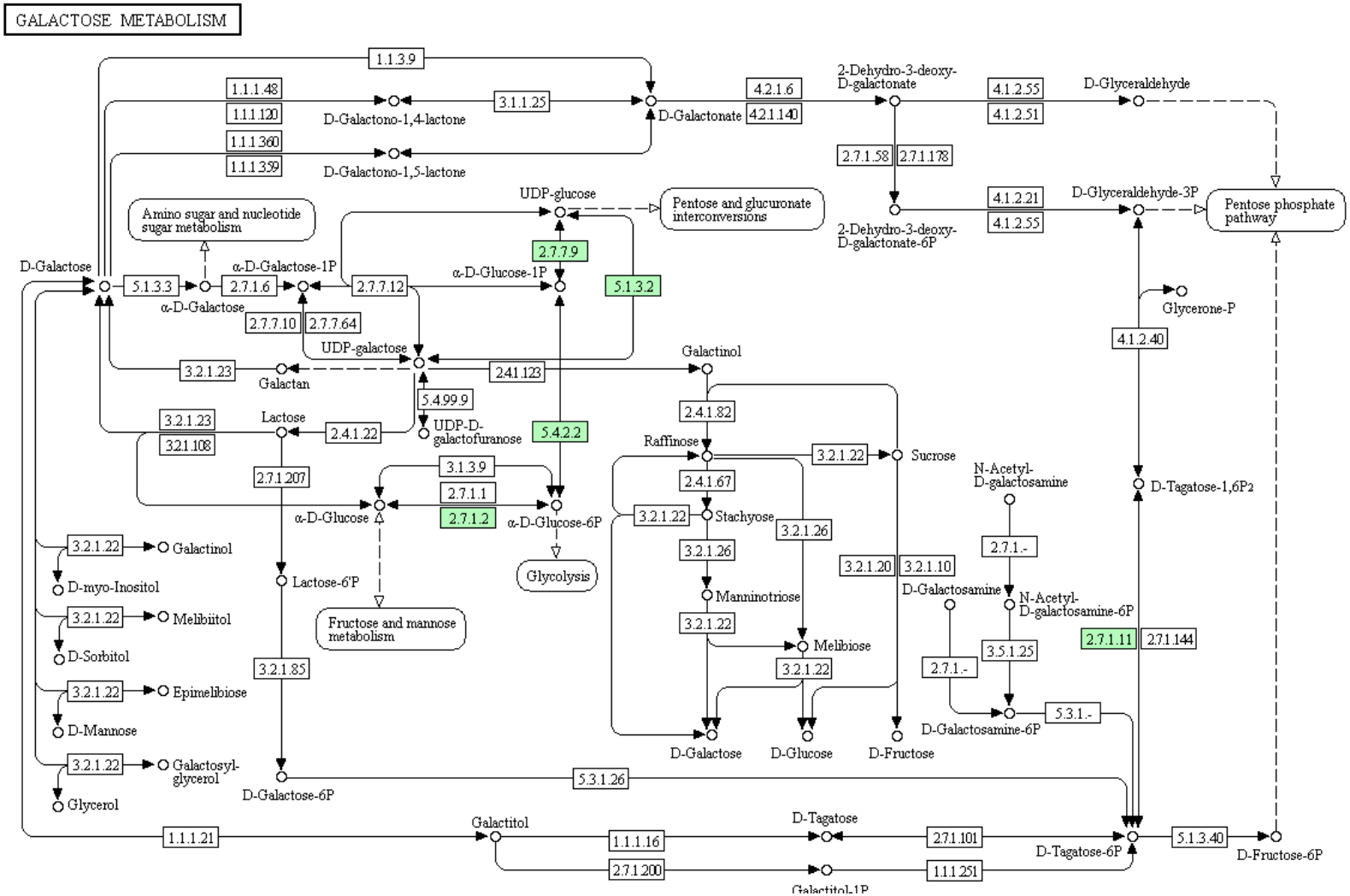

Figure 6. KEGG pathway for galactose utilisation associated genes *in Polaromonas naphthalenivorans*. The strain lacks the essential kinase/PEP transferase for galactose phosphorylation and entry into glycolysis or tagatose pathways.

Conclusions

Recently, significant research interest around *Polaromonas* sp. has arisen due the metabolic diversity of the genus (Yagi et al., 2009; Willems et al., 2014; Gonzalez-Martinez et al., 2018). The capacity to develop biotechnological opportunities may emerge, but it will be crucial to cultivate strains effectively to facilitate the study of functional capabilities. Further investigations on new strains will be important to fully profile the capabilities of the genus and it remains to effectively isolate cultures from the IASBR system, where *Polaromonas* dominated under low temperature operation. In addition, elucidation of the nitrogen and phosphorus removal pathways should be continued and the stressor tests for functional characterization conducted herein may assist. Finally, optimisation of isolate growth, particularly in lactose rich settings, requires further investigation in particular to assist with the development of bioaugmentation strategies for bioreactors dependent on *Polaromonas* genus activities for high performance nutrient remediation.

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Appendix 1

Dominance of the genus *Polaromonas* in the microbial ecology of an Intermittently Aerated Sequencing Batch Reactor (IASBR) treating dairy processing wastewater under varying aeration rates.

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Summary

In this Research Communication we investigate potential correlations between key bacterial groups and nutrient removal efficiency in an Intermittently Aerated Sequencing Batch Reactor (IASBR) treating synthetic dairy processing wastewater. Reactor aeration rates of 0.6 and 0.4 litre per minute (LPM) were applied to an 8 litre laboratory scale system and the relative impacts on IASBR microbial community structure and orthophosphate (PO_4-P) and ammonium (NH_4-N) removal efficiencies compared. Aeration at 0.6 LPM over several sludge retention times (SRTs) resulted in approximately 92% removal efficiencies for both PO_4-P and NH_4-N. Biomass samples subjected to next generation sequencing (NGS), 16S rRNA profiling revealed a concomitant enrichment of *Polaromonas* under 0.6 LPM conditions, up to ~50% relative abundance within the reactor biomass. The subsequent shift in reactor aeration to 0.4 LPM, over a period of 3 SRTs, resulted in markedly reduced nutrient removal efficiencies for PO_4-P (50%) and NH_4-N (45%). An 85.7% reduction in the genus level relative abundance of *Polaromonas* was observed under 0.4 LPM aeration conditions over the same period.

Introduction

Liquid milk can be processed into various downstream products such as milk powder, cheese and ingredients, resulting in high wastewater volumes. A typical European dairy processing plant produces approximately 500 m^3 of wastewater daily (Demirel *et al.*, 2005). Such wastewaters represent a potential environmental risk (e.g eutrophication) due to high nutrient loads, requiring extensive treatment prior to discharge into receiving water bodies. Traditional biological treatments are often applied in dairy processing wastewater remediation but typically require chemical precipitant application and potentially high energy inputs. Intermittently aerated sequencing batch reactor (IASBR) technology has been previously described in the treatment of wastewater (Pan *et al.*, 2013) as a cost efficient, sustainable wastewater treatment approach. The technology relies on biomass adaptation via multiple,

alternating anaerobic/aerobic periods to achieve efficient removal of organic carbon (BOD), nitrogen (N) and phosphorus (P). The advantages of this system include a single operational reactor with reduced energy and chemical precipitant inputs when compared with traditional biological approaches.

In biological wastewater treatment technologies, the performance of the system is dependent on the action of key microorganisms for nutrient remediation. Biological reactor operational parameters play a major role in the selection and stability of such microorganisms. Understanding the relationship between the microbial communities in biological reactors and treatment performance has been found critical in the improvement/optimisation of these technologies (Pholchan *et al*., 2009, Ferrera *et al.*, 2016). Research to date into the microbiology underpinning IASBR systems has been limited to investigation of the spatial distribution of nitrite oxidizing and ammonia oxidizing bacteria, respectively (Pan *et al.,* 2013). The current study applied NGS technology to fully characterize the microbial populations contributing to varying nutrient remediation performances of a laboratory scale IASBR system.

Material & Methods

Laboratory-scale IASBR set-up and sample collection

The IASBR reactor was seeded with municipal sludge and operated at 11^{o}C in 12 hour cycles with varying aeration rates (0.6 and 0.4 LPM). The reactor had a 4 day hydraulic retention time (HRT) and 20 days solid retention time (SRT). Physicochemical parameters were tested following standardized analytical procedures (APHA 2005) and the manufacturer's instructions for a Konelab 20 Nutrient Analyser (Thermo Scientific). Biomass sampling for NGS analyses were chosen during 0.4 and 0.6 LPM aeration periods and reflection variations in reactor nutrient remediation performance. Samples were frozen at -20°C until further analyses.

Molecular analyses of bacterial community composition and bioinformatics

In order to ensure sufficient biomass for optimal nucleic acid extraction, sludge was centrifuged prior to DNA extraction. Genomic DNA from mixed liquor sludge samples was extracted using PowerSoil Isolation Kit (MOBIO Laboratories) according to the manufacturer's instructions. Each sample was amplified using polymerase chain reactor (PCR) technique in triplicate to ensure representative sampling and sequenced by an external service provider (454 GS FLX+ pyrosequencing, MACROGEN, Seoul). Sequences were corrected using Acacia (Bragg *et al*, 2012) and processed using Quantitative Insights Into Microbial Ecology (QIIME) pipeline (Caporaso *et al.,* 2010). Taxonomy was assigned to each operational taxonomic unit (OTU) at 97% similarity in an open-reference OTU picking process implemented in QIIME. Taxonomy graphs were filtered at 3% cut-off.

Results and discussion

Genus level microbial diversity and percentage removal of ammonia (NH_4-N) and orthophosphate (PO_4-P) during each of the sampled SRTs are shown in Figure 1. Under 0.6 LPM aeration conditions (SRTs 6-8) ~92% removal for both NH_4-N and PO_4-P was observed. This coincided with a clear dominance, up to 51.4% abundance, at genus level by *Polaromonas* (figure 1 B). The competitive capacity of this genus appeared to be confined to the specific physicochemical conditions arising under 0.6 LPM aeration. The subsequent shift to 0.4 LPM resulted in a rapid reduction in the abundance of *Polaromonas* representatives (11% in SRT 9) and an increase of the bacterial diversity from SRT 10 compared with the dominance of a few groups of bacteria under 0.6 LPM aeration. During the 0.4 LPM period lower removal efficiencies were detected with higher impact in PO_4-P removal, $\leq$60% removal efficiency (figure 1 C). *Polaromonas*, a member of the family *Comamonadaceae*, has previously been identified within the microbial community structure of an activated sludge system treating municipal wastewater operated at 5 °C (He *et al.*, 2016). Evidence from the literature shows a positive correlation between several

members of the *Comamonadaceae* family and biological nutrient removal (BNR) (Willems, 2014, Ge *et a*l., 2015). The strong dominance of *Polaromonas* during the optimal nutrient removal performance in the IASBR suggests that the genera may contribute significantly to BNR processes. Other microbial groups with established links to BNR, such as *Flavobacterium* and *Thauera,* (Weissbrodt *et al*, 2014) were also observed in this study. However, the dominant ecological shifts were related to *Polaromonas* and other members of the family *Comamonadaceae* ("Other *Comamonadaceae*" group). Further work is required to assess the functional contribution of *Polaromonas* enrichment within the IASBR and, by extension, other biological treatment approaches.

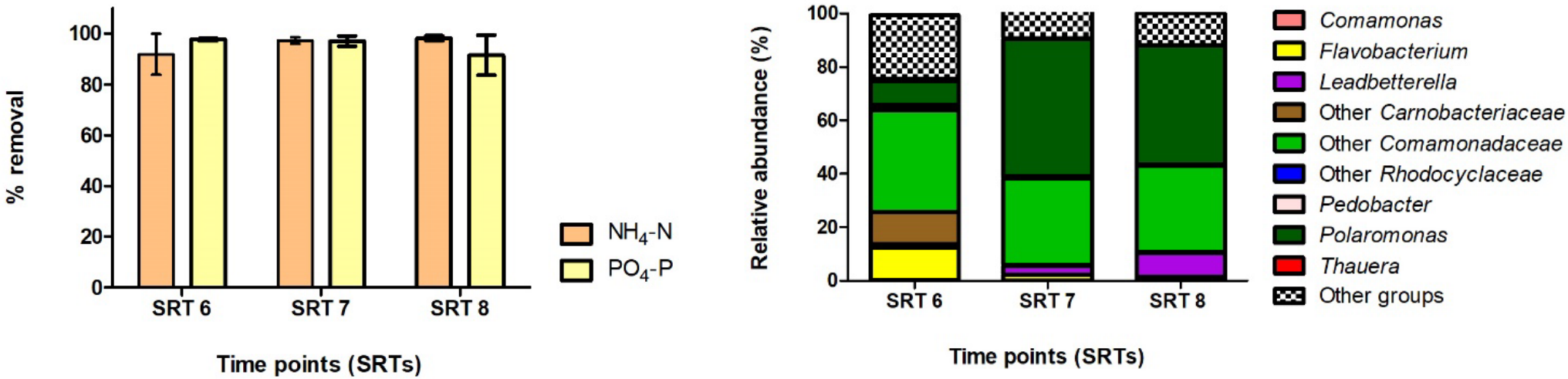

Figure 1a. Removal efficiencies (%) of NH_4-N and PO_4-P and respective solid retention times (SRTs) during 0.6 LPM and bacterial composition at genus level.

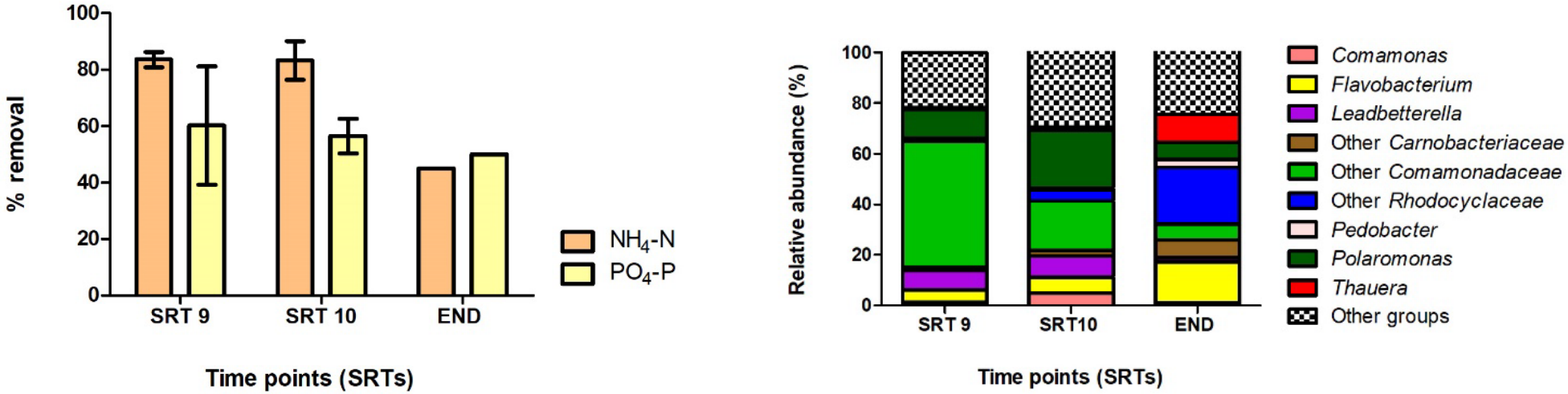

Figure 1b. Removal efficiencies (%) of NH_4-N and PO_4-P and respective solid retention times (SRTs) during 0.4 LPM and bacterial composition at genus level. “END” corresponds to the final day of bioreactor operation at the conclusion of SRT10.

Conclusion

In summary, IASBR application to synthetic dairy processing wastewater appears to be a promising technology capable of efficient remediation of high nutrient loads. Minor alterations in IASBR aeration rates (0.6 vs. 0.4 LPM) had significant impacts on reactor performance. These impacts were reflected in changes within the microbial ecology profiles, which suggest a dependence of IASBR performance on specific bacterial community structures. The analyses suggest the potential importance of the genus *Polaromonas* in the system under investigation, which has not been previously reported.

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Appendix 2

Supplementary material Chapter 2

Table S1. Correlation of taxonomy (up to family level) and relative contributions to genes of interest: ppk, ppx, phaC, nirK, norB and nosZ [a].

Relative Abundance Rank	Taxonomy	Total Relative Abundance (%)	ppk (%)	ppx (%)	phaC (%)	nirK (%)	norB (%)	nosZ (%)
1	*Comamonadaceae*	38.2	30.90	30.36	58.33	5.10	50.12	31.13
2	*Order SC-I-84*	10.4	0.88	0.88	1.71	-	1.75	-
3	*Carnobacteriaceae*	8.8	-	-	-	-	-	-
4	*Rhodocyclaceae*	8.2	6.19	6.71	13.96	17.33	12.28	22.77
5	*Flavobacteriaceae*	8	11.61	10.70	-	20.70	8.03	8.27
6	*Thiotrichaceae*	4.1	3.16	3.14	6.11	-	-	-
7	*Saprospiraceae*	3.6	6.68	3.15	-	-	-	16.93
8	*Cytophagaceae*	2.4	2.27	4.31	-	-	-	0.51
9	*Xanthomonadaceae*	2.2	10.37	10.17	9.90	34.63	15.89	0.71
10	*GZKB119*	2	0.89	0.89	-	-	-	-
11	*Streptococcaceae*	1.7	-	-	-	-	-	-
12	*Weeksellaceae*	1.5	2.57	2.55	-	9.48	4.04	0.92
13	*Erysipelotrichaceae*	1.1	-	-	-	-	-	-
14	*Chitinophagaceae*	0.9	4.44	2.96	-	-	-	7.73
15	*Burkholderiaceae*	0.7	0.41	0.41	0.80	-	-	-
16	*Sphingobacteriaceae*	0.6	4.44	4.12	-	-	-	-
17	*Acidaminobacteraceae*	0.6	-	0.41	-	-	-	-
18	*Porphyromonadaceae*	0.4	1.03	0.71	-	-	-	-
19	*Peptococcaceae*	0.4	-	1.30	1.27	-	-	-
20	*Rhodobacteraceae*	0.4	1.45	2.88	1.41	6.85	2.65	6.53
21	*Order Bacteroidales*	0.3	1.29	1.14	-	-	-	-
22	*SB-1*	0.3	1.11	1.10	-	-	-	-
23	*Cryomorphaceae*	0.3	0.91	1.16	-	-	-	-
24	*Enterococcaceae*	0.3	-	-	-	-	-	-
25	*Order SBla14*	0.3	0.37	0.37	-	-	0.73	-

26	*Class Betaproteobacteria*	0.2	0.38	0.38	0.74	-	0.76	-
27	*Order Burkholderiales*	0.2	0.31	0.31	0.58	0.13	0.62	-
28	*Pseudomonadaceae*	0.2	0.29	0.28	0.55	-	-	-
29	*Propionibacteriaceae*	0.1	0.56	0.55	-	2.86	1.11	-
30	*BA008*	0.1	0.41	0.41	-	-	-	-
31	*Marinilabiaceae*	0.1	0.33	0.24	-	-	0.17	-
32	*Order Sphingobacteriales*	0.1	0.37	0.56	-	-	-	-
33	*Aerococcaceae*	0.1	-	0.13	-	-	-	-
34	*Lactobacillaceae*	0.1	-	0.10	-	-	-	-
35	*Leuconostocaceae*	0.1	0.13	0.38	-	-	-	-
36	*Lachnospiraceae*	0.1	1.24	0.31	-	-	-	-
37	*Peptostreptococcaceae*	0.1	0.13	0.31	-	-	-	-
38	*Class TSBW08*	0.1	-	0.44	-	-	-	-
39	*Order Ellin6067*	0.1	0.27	0.26	0.26	1.36	0.53	-
40	*Order MND1*	0.1	0.40	0.39	0.77	-	0.79	2.02
41	*Enterobacteriaceae*	0.1	0.42	0.76	-	-	-	-
42	*Sinobacteraceae*	0.1	0.94	0.94	1.83	-	-	-
43	*Acholeplasmataceae*	0.1	-	-	-	-	-	-

Appendix 3

Supplementary material Chapter 3

Table S1: Alpha diversity indices and Good's coverage values for bioreactor samples.

Location	Sample code	ACE	Chao 1	Unique OTUs
IASBR 1	R1 (d 81)	403.0	403.0	403.0
IASBR 1	R1 (d 94)	374.0	374.0	374.0
IASBR 1	R1 (d 112)	324.0	324.0	324.0
IASBR 1	R1 (d 140)	356.0	356.0	356.0
IASBR 1	R1 (d 167)	358.0	358.0	358.0
IASBR 2	R2 (d 81)	656.3	689.2	490.0
IASBR 2	R2 (d 94)	742.9	687.4	556.0
IASBR 2	R2 (d 112)	656.5	681.7	541.0
IASBR 2	R2 (d 140)	704.4	791.2	509.0
IASBR 2	R2 (d 167)	451.0	451.0	451.0

Table S2: Differential abundances at genus level and significance (UP = What increases in IASBR 2 that decreased in IASBR 1) (DOWN = What decreased in IASBR 2 that increases in IASBR 1).

Taxonomy(UP)	padj	Aprox. Fold Change Increase
Lentimicrobiaceae	0.0004	34.5819
Fusibacter	0.0009	30.6675
Paludibacter	0.0045	25.9769
Rhodobacteraceae	0.0009	24.9870
Desulfosporosinus	0.0019	16.4793
Lactococcus	0.0079	13.7544
Leadbetterella	0.0064	11.7426
Enterococcus	0.0146	9.7323
Hydrogenophaga	0.0073	6.9209

Taxonomy (DOWN)	padj	Aprox. Fold Change Decrease
Polaromonas	2.42E-16	149.6254909
Comamonadaceae	7.02E-05	13.08462545
Rhodocyclaceae	0.006635334	12.66329595
Bacteroidetes_D_2__WCHB1-32	0.004419785	7.807785225
Luteimonas	0.02518588	7.379934582

	R1 (d 81)	R1 (d 94)	R1 (d 112)	R1 (d 140)	R1 (d 167)	R2 (d 81)	R2 (d 94)	R2 (d 112)	R2 (d 140)	R2 (d 167)
R1 (d 81)		0.79	0.77	0.71	0.67	0.67	0.61	0.47	0.50	0.55
R1 (d 94)	0.79		0.82	0.82	0.71	0.60	0.56	0.53	0.54	0.63
R1 (d 112)	0.77	0.82		0.78	0.73	0.53	0.50	0.46	0.53	0.53
R1 (d 140)	0.71	0.82	0.78		0.81	0.57	0.54	0.48	0.49	0.62
R1 (d 167)	0.67	0.71	0.73	0.81		0.53	0.50	0.44	0.47	0.52
R2 (d 81)	0.67	0.60	0.53	0.57	0.53		0.82	0.73	0.73	0.59
R2 (d 94)	0.61	0.56	0.50	0.54	0.50	0.82		0.79	0.78	0.59
R2 (d 112)	0.47	0.53	0.46	0.48	0.44	0.73	0.79		0.83	0.58
R2 (d 140)	0.50	0.54	0.53	0.49	0.47	0.73	0.78	0.83		0.65
R2 (d 167)	0.55	0.63	0.53	0.62	0.52	0.59	0.59	0.58	0.65	

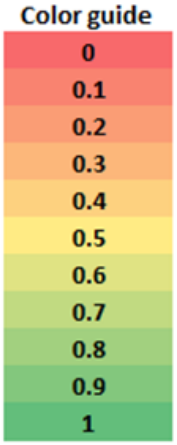

Figure S1: Community similarity. Heat map based on Morisita similarity index among bioreactor samples. Higher scores (dark green = 1) indicate more similarity, while lower scores (red = 0) indicate more difference.

Family	Genus	Contribution to dissimilarity (%)
Comamonadaceae	Other	33.3
Comamonadaceae	*Polaromonas*	8.2
Comamonadaceae	*Hydrogenophaga*	6.0
Saprospiraceae	Uncultured	5.7
Carnobacteriaceae	*Atopococcus*	5.6
Cytophagaceae	*Leadbetterella*	4.3
Rhodobacteraceae	Other	3.6
Flavobacteriaceae	*Flavobacterium*	3.5
Lentimicrobiaceae	Other	2.0
Blastocatellaceae (Subgroup 4)	Uncultured	1.8
Xanthomonadaceae	*Aquimonas*	1.5
Saprospiraceae	*Haliscomenobacter*	1.4
Xanthomonadaceae	*Luteimonas*	1.3
Rhodocyclaceae	*Thauera*	1.2
OPB56	Uncultured bacterium	1.1

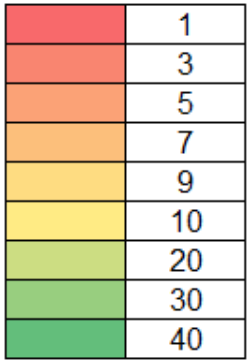

Figure S2: Similarity percentage (SIMPER) analysis. The heat map plot represents the genera that contributed the most (≥ 1% contribution) to the dissimilarity between bioreactor community structure. Data shown in the heat map was used to analyse the correlation between statistically significant operational parameters (Chapter 3, Table 3) over the ecological composition of IASBRs by means of redundancy analyses (RDA) (Chapter 3, Figure 4).